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The Activation of Bee Venom

Phospholipase A₂

by Denis Drainas B.Sc.

A Thesis Submitted to the University of Glasgow

for the Degree of Doctor of Philosophy

October 1978

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to my parents

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ACKNOWLEDGEMENTS

I wish to thank my supervisor Dr. A. J. Lawrence for his help, encouragement and advice during the course of this thesis; Professor A.S.G. Curtis, Dr. G.R. Moores for helpful discussion and advice and Mr. J. Connelly for excellent technical assistance.

I also wish to extend my thanks to all the rest of the academic and technical staff of the Department of Cell Biology, University of Glasgow, for their friendship and collaboration contributing to an excellent environment during the course of this work.

I gratefully wish to thank my parents who supported me financially and mentally during all the years of my studies, especially during the course of my thesis.

Abbreviations

CMC	Critical micelle concentration
GPC	Glycerophosphorylcholine
NMR	Nuclear magnetic resonance
TLC	Thin layer chromatography
PLA ₂	Phospholipase A ₂
LEC	Lauroyl ethyl carbonate
DMMA	Dimethyl maleic anhydride
EDTA	Ethylenediaminetetraacetic acid

SUMMARY

The kinetic behaviour of Bee venom phospholipase A₂ was studied using both long and short chain substrates in the presence and absence of organic solvents. The activity was controlled by a variety of activators and in particular the behaviour in dilute organic solvents was dominated by fatty acid activation.

The enzyme was activated irreversibly by a variety of acylating agents derived from long-chain fatty acids. The most effective derivatives were azides and imidazolides and the reactivity was proportional to the hydrocarbon chain length, in contrast to the general chemical reactivity. Oleoyl and linoleoyl imidazolidine were shown to be the most effective activators and the oleoyl derivative gave full activation corresponding to addition of one acyl residue per protein molecule.

Studies of maximally activated enzyme showed that earlier models in which activation was proposed to rely on fatty acid modification of the substrate surface could be ruled out. Activation does not appear to be due to modification of the rate at which the enzyme penetrates the substrate surface but it is sensitive to the physico-chemical properties of the substrate. A model is proposed whereby allosteric activation of the enzyme is communicated to the active site of the protein by an interaction involving the acyl side chains of the substrate.

Activation is calcium dependent and at low calcium concentrations the activation factors exceed 100-fold. Furthermore the same experiments produced evidence that the enzyme has two sites for calcium and the activation by acylating agents substitutes the second weakly reactive calcium.

The covalently activated enzyme causes lytic damage to the rabbit erythrocytes where the unactivated enzyme has very little effect. Therefore this type of activation could be of importance in vivo.

Chapter I : Introduction

1.1 Phospholipase A enzymes

Phospholipase A enzymes catalyze the hydrolysis of one fatty acid residue from glycerophospholipids which are the main structural elements of biological membranes.

These enzymes are widely distributed and occur in the venoms of snakes, bees, scorpions, and probably in all mammalian tissues.

The widespread occurrence of Phospholipase A activity in a variety of biological membranes has created much interest in the nature and function of these enzymes. The enzyme has been detected in mitochondria (Waite et al., 1969), microsomes (Waite and Van Deenen, 1967), plasma membranes (Newkirk and Waite, 1971), and lysosomes (Blaschko et al., 1967).

The importance of the Phospholipase A₂ (EC 3.1.1.4) for studies of membrane fusion (Lucy, 1970), modulation of cell interactions (Curtis et al., 1975), and in the release of prostaglandin precursor arachidonic acid from phospholipids (Su-Chen et al., 1976), coupled with its widespread use in solubilizing membrane-bound proteins and in studying membrane structure has led to the purification of the phospholipase A₂ from many sources. Excellent reviews of the action of phospholipases have been written by Habermann (1972), Gatt and Barenholz (1973), and Van den Bosch (1974).

Fischer et al. (1967) established that these enzymes occur in cell membranes and form part of a cyclic system for the deacylation and reacylation of glycerophospholipids in the membranes. They

demonstrated that intact erythrocytes show both acyltransferase (reacylation) and lysophospholipase (deacylation) reactions but when the cells are rich in ATP (fresh cells) there was more acyltransferase activity while aged cells have more lysophospholipase activity. The stimulation of acyltransferase and inhibition of lysophospholipase activity is entirely due to the intracellular ATP. Exogenous ATP does not increase the acyltransferase activity of fresh cells. Furthermore they found inhibition of acylating activity in antibody-coated sheep erythrocytes and also treatment with some membrane bound drugs increases the lysophospholipase activity. These results showed that in the intact cell the synthesis of lecithin might be limited and regulated by the availability of intracellular ATP and membrane bound enzymes participate in lysolecithin metabolism.

Evidence for a biological role of this deacylation and reacylation cycle was provided by Curtis et al. (1975a,b). Curtis et al. (1975a,b) showed that lysolecithin produced by the action of phospholipase A₂ in plasmalemma membrane of neural retinal cells is responsible for the inhibition of cell adhesions. In conditions which would be expected to stimulate reacylation of lysolecithin in the plasmalemma membrane, i.e. incubation of the cells in the external presence of CoA, ATP and a fatty acid lead to recovery or maintenance of adhesion. Furthermore they showed that plasmalemma contains the enzymes to effect the turnover.

1.2 Bee Venom

Bee venom contains a considerable number of pharmacologically or enzymatically active components but its complete biochemical and

pharmacological analysis has nevertheless been feasible.

Among the low molecular weight agents in bee venom histamine predominates. This substance is present in a low concentration, 0.1 to 1.5 percent (Schachter and Thain, 1954). Recently, dopamine and noradrenaline have been identified as constituents of bee venom reservoir in vivo, but not the secreted, dried venom (Owen, 1971).

Application of electrophoretic, chromatographic, and gel-filtration procedures, together with pharmacological and biochemical analysis, led to the differentiation of two enzymes, namely phospholipase A and hyaluronidase, from a series of toxic polypeptides: the hemolyzing melittin the neurotoxic apamin and a mast cell degranulating peptide. This thesis deals with the properties of the enzyme phospholipase A, but it is necessary to discuss the properties of melittin because the action of these two compounds may be closely linked.

1.2.1 Melittin

The polypeptide melittin, with respect to weight (it is 50 percent of dry venom) and activity, is the main toxin of bee venom.

Two properties of the melittin molecule gave hints of some molecular characteristics of possible biological significance. First, it is strongly basic (Neumann and Habermann, 1954) like the other peptides of bee venom. A second peculiarity lies in the strong surface activity of melittin. The peptide decreases the interfacial tension between air and salt solutions to a degree comparable with the

hemolysins lysolecithin or digitonin (Habermann, 1972), and increases the permeability of erythrocytes and other cells.

Determination of the amino acid sequence eventually gave the key to the understanding of the physicochemical and most of the pharmacological properties of the peptide (Habermann, 1972).

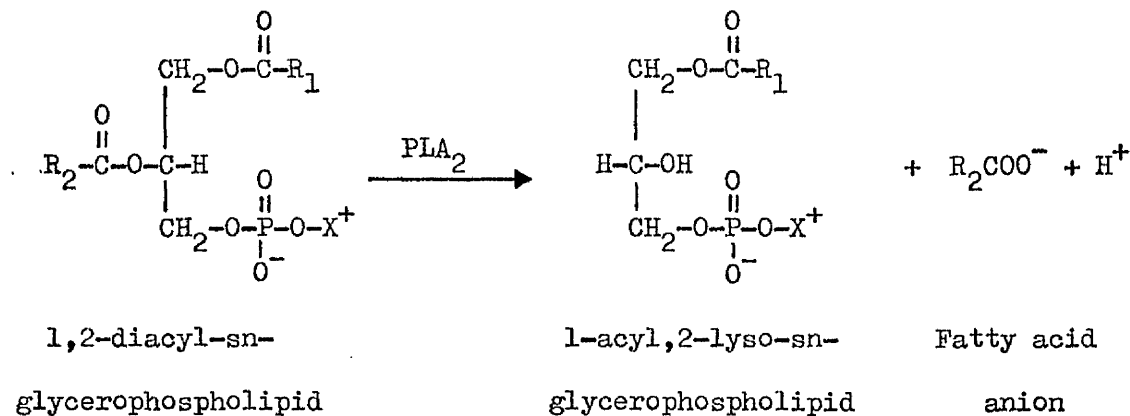
Studies with lipid monolayers indicated that melittin had a very high affinity for air-water interface and even more for the lipid-water interface.

Hemolysis is the commonest model system for studying the action of melittin, but melittin has been effective on nearly every other pharmacological system tested, provided that it is not bound by compounds present in nutrient solutions (such as blood) before reaching the sensitive structures. Melittin damages not only erythrocytes but also leukocytes and their lysosomes with subsequent release of enzymes. However, the mechanism of melittin hemolysis is very different to that of other detergents, such as lysolecithin or digitonin. The best evidence for a specific attack by melittin stems from subsequent appearance of the erythrocyte ghosts. Lysolecithin is a typical "solubilizer" where high concentrations of erythrocyte ghosts are totally dissolved. Digitonin, reacting with the erythrocyte cholesterol, produces clodlike changes when applied in high concentrations. Melittin, in contrast, renders the ghosts shrunken and more strongly contrasting in the dark field, phase contrast, or electron microscope.

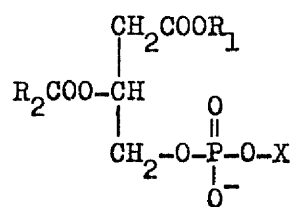
1.2.2 Phospholipase A

Shipolini et al. (1971, 1974a,b) reported the complete structure (primary sequence) of Phospholipase A₂ from the venom of the common European honey bee Apis mellifica.

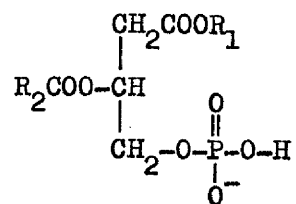
The Bee venom Phospholipase A₂, a small cationic protein of a molecular weight of 14,629, pI = 10.5 \pm 1.0 and four disulphide bridges, catalyses the hydrolysis of the fatty acid esterified to the number two position of 1,2-diacyl-sn-glycerophospholipids.



The enzyme attacks structural phospholipids, and as phospholipids are integral parts of biological membranes, their loss could cause failure of cellular functions. However the damage to the membrane is essentially produced by the reaction products. One group of the products consists of the lysophospholipids. Their surfactant character stems from the combination between the hydrophobic aliphatic acyl chain and the hydrophilic phosphorylated base. Such molecules not only lower the surface tension of water and form micelles, but also solubilize cholesterol, lecithin and particulate tissue fractions. Like melittin, lysolecithin is a "structural poison". In addition, the fatty acid product may be toxic and some pharmacological



a.



b.

Fig. (1) a. The general formula of Glycerophospholipids

R_1, R_2 , fatty acids

"X", base

b. Phosphatidic acid Glycerophospholipids

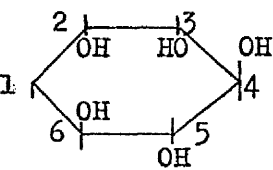
"X"	Class	
-H	Phosphatidic acid glycerophospholipids	
-CH ₂ CH ₂ N ⁺ (CH ₃) ₃	Choline	"
-CH ₂ CH ₂ N ⁺ H ₃	Ethanolamine	"
$\begin{array}{c} \text{N}^+\text{H}_3 \\ \\ -\text{CH}_2\text{CHCOO}^- \end{array}$	Serine	"
	Inositol	"

Table (1) The most common classes of glycerophospholipids
according to the group "X".

effects of lyso compounds or phospholipase A can be imitated by higher concentrations of fatty acids.

Phospholipase A activity depends on wide variety of factors including the exact chemical and physico chemical nature of the substrate, the temperature, the presence of metal ions and of a variety of amphipathic activators.

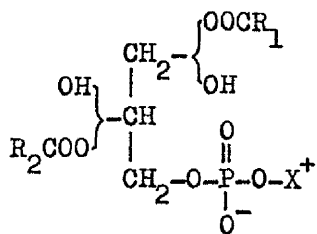
In comparison with most other enzyme systems the substrates for phospholipases are extremely complex both in chemical structure and in the physico chemical forms which these structures adopt. Because this is of great importance in understanding the control of enzyme activity the properties of the relevant glycerophospholipids must be discussed in detail.

The best characterized phospholipases A_2 are from - Bee venom (Shipolini et al., 1971, 1974), from pig pancreas (de Haas et al., 1968), from the Crotalus adamanteus venom (Wells and Hanahan, 1969), and from the cobra Naja-Naja venom (Deems and Dennis, 1975).

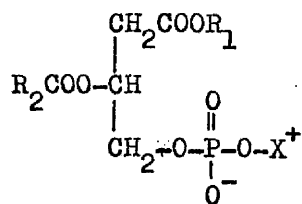
These enzymes are activated by many different compounds, and the present thesis deals with the activation of bee venom phospholipase A_2 by fatty acids and other related compounds.

1.3 Glycerophospholipids

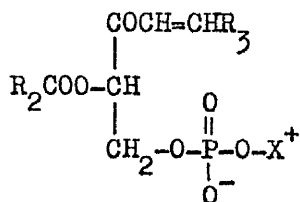
The parent compound of the family of glycerophospholipids is Phosphatidic acid (Fig. 1) from which the other compounds are derived by addition of "base" groups "x" to the phosphate. Table (1) shows



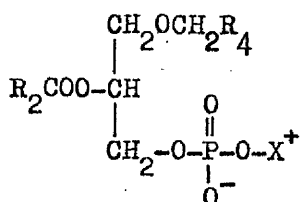
a



b

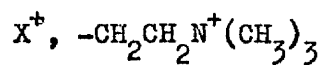


c



d

Fig. (2) The naturally occurring types of choline glycerophospholipids.



a, 1-acyl or 2-acyl Lyso PC

b, 1,2 diacyl PC

c, 1-alk-1-enyl, 2-acyl CP

d, 1-alkyl, 2-acyl derivative

the most common classes according to the group "x".

Glycerophospholipids are among the main components of cellular and subcellular membranes. In mammalian tissues the Choline containing phospholipids are most abundant and have attracted the greatest interest. It is particularly relevant that a highly convenient source of phosphatidyl choline is present in avian egg yolk. Hence the common name Lecithin frequently used, applied to this family of compounds.

At least four types of choline glycerophospholipids occur naturally:

- (a) monoacyl derivatives (1- or 2-acyl-sn-glycero-3-phosphorylcholine, lysophosphatidylcholine or lysolecithin, lyso-PC);
- (b) diacyl derivatives (1,2-diacyl-sn-glycero-3-phosphorylcholine, phosphatidylcholine or lecithin PC);
- (c) alk-1-enyl-acyl derivatives (1-alk-1'-enyl-2-acyl-sn-glycero-3-phosphorylcholine or choline plasmalogen CP);
- (d) alkyl, acyl derivatives (1-alkyl-2-acyl-sn-phosphorylcholine).

Of these four PC occurs most widely and in greatest abundance (Fig. 2).

The nitrogen-base choline has a pK near 13 and the phosphate moiety has a pK of ca. 2.0 thus the PC is zwitterionic at neutral pH.

For PC from many sources saturated fatty acids (16:0 and 18:0) predominate at the 1-position and unsaturated fatty acids (monoenoic 16:1, 18:1, dienoic 18:2 and polyenoic 20:4 etc) predominate

at the 2-position. Generally, animal phospholipids contain mostly fatty acids between chain length 16 and 20 with palmitic (16:0), stearic (18:0), linoleic (18:2) and arachidonic (20:4) predominant. Plant leaf phospholipids have a more limited range with very few fatty acids greater than C₁₈.

Almost without exception the discovery and isolation of the above phospholipids depended on differences in solubility exhibited by the natural phospholipids, or by the specific salt forms in various organic solvents. In the early 1950's a number of developments occurred which greatly facilitated the isolation and analysis of phospholipids. These developments included the application of silicic acid column chromatography and silica-impregnated paper to separations of intact lipids and the development by Dawson of a mild alkaline hydrolysis procedure for the release of water-soluble phosphodiester (from phospholipids) which could be separated by two dimensional paper chromatography. The availability of the above techniques and their refinement to a very high level and the more recent application to polar lipids of thin-layer chromatography (TLC) has permitted the separation and characterization of many new phospholipids over the last years.

1.4 Phospholipid Micelles

In aqueous systems, the polar phospholipids exist singly in true solution below a certain concentration called the critical micelle concentration (CMC). Above this concentration, phospholipids,

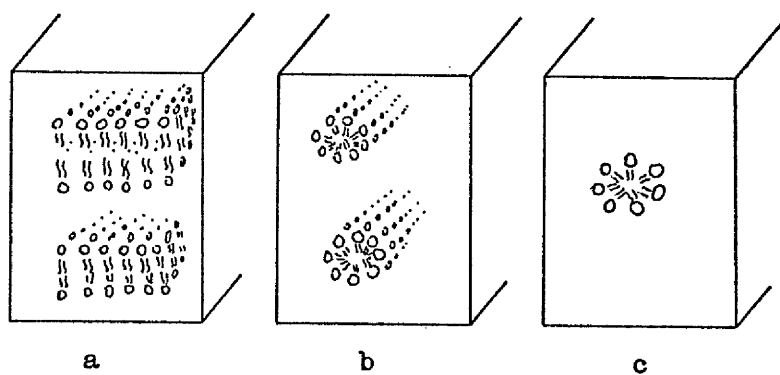


Fig. 3 Micelle forms

- a. Lamellar
- b. Cylindrical
- c. Spherical

which have a polar ("phosphoryl-x") end and a non-polar (fatty acid) end, tend to form aggregates or micelles which are arranged with the polar ends in the aqueous environment.

The term micelle is generally used for the spherical or cylindrical aggregates (Fig. 3) which are formed when the phospholipid content is fairly low relative to the aqueous phase. When the water content is low, on the other hand, the phospholipids can exist in a number of liquid crystalline phases (liposomes). These spontaneous molecular rearrangements are analogous to the lyotropic mesomorphism of soaps of long-chain aliphatic and other amphipathic molecules. The predominant phase depends on such factors as the water content, temperature and the type of lipid involved. For example, a zwitterionic phospholipid such as phosphatidyl choline more readily forms sheet-like arrays or lamellae (Fig. 3) because the positive and negative charges on the headgroup balance each other and there is therefore no repulsion between them. This allows the hydrocarbon chains to come close together to achieve the maximum degree of hydrophobic bonding.

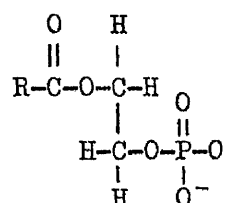
The tendency of phospholipids to assume this hydrated lamellar configuration is also related to the transition temperature (melting of hydrocarbon chains). Thus, natural phospholipids, which are usually above this transition at ambient temperatures, will form such structures spontaneously. Synthetic phospholipids with saturated acyl chains will also form similar structures, if the temperature is raised close to transition point of the dry compounds.

1.5 The specificity of Phospholipase A₂ on the substrate stereochemical configuration

Bee venom phospholipase A₂, like all the phospholipases A₂ from various sources, hydrolyses only the fatty acid from 1,2-diacyl-sn-3-glycerophospholipids.

Van Deenen and de Haas (1963) reported the substrate specificity of the Crotalus adamanteus enzyme and they established the following points:

- (a) The enzyme is stereospecific and hydrolyses only the fatty acid from 1,2-diacyl-sn-3-glycerophospholipids.
- (b) β-Lecithins were hydrolyzed in a stereospecific manner also, with only one isomer being attacked.
- (c) Glycol analogues were also hydrolyzed.
- (d) They concluded that the minimum structural requirements for an active substrate were:



Furthermore Bird et al. (1965) demonstrated that the phospholipase A₂ from Crotalus adamanteus venom has different affinity for structurally isomeric L-α-lecithins (1,2-diacyl-sn-3-phosphatidylcholines). The 1-oleoyl-2-butyryl lecithin was found to be more readily attacked than 1-butyryl-2-oleoyl lecithin.

Bonsen et al. (1972) demonstrated the substrate specificity of pancreatic phospholipase A₂ for 1,2-acyl-sn-3-phosphatidylcholines and showed the inhibitory effect of the opposite configuration lecithins (D-lecithins) and other modified lecithins which proved to be pure competitive inhibitors for the enzyme.

1.6 Effect of the Physical state of the Substrate

A reaction in which an enzyme acts on a phospholipid usually occurs at lipid-water interfaces rather than in the bulk phase of an aqueous solution. Such enzyme-lipid interactions are therefore examples of heterogenous catalysis. The various procedures used for "solubilization" of the complex lipids, such as addition of detergents or organic solvents, emulsification, sonic oscillation, do not yield true molecular solutions in water. Aggregates are formed which may be organized as micelles, mixed micelles, bilayered or multi-bilayered liposomes and monolayers at an air-water interface. Common to all of these structures is the orientation of lipid molecules at an interface. The significance of this fact is severalfold. First it permits study of a new parameter of enzyme catalysis, namely the influence of the "quality of interface" on enzyme action (Verger et al., 1973). This quality is presumed to arise from the nature of lipid orientation or energy at the interface, or both. Secondly, lipolytic reactions might provide a model system for the study of the physical and chemical aspects of the interaction of lipids and proteins in membranes.

Kinetic analysis of enzymes acting at interfaces can not be

treated by the classical Michaelis-Menten theory, because of the two-dimensional state of the substrate involved. In order to overcome this problem Verger et al. (1973) proposed a simple model to describe kinetically the action of soluble enzymes at interfaces. This model consists basically of two successive equilibria. The first step describes the reversible penetration of a water-soluble enzyme (E) into an interface. When the enzyme has penetrated, it is in a more favourable energetical state (E'). After this first step of penetration follows a second equilibrium giving a kind of interfacial "Michaelis-Menten" complex. This complex denoted E'S, is formed by the combination of a single substrate molecule with the penetrated enzyme. This is the equivalent in two dimensions of the classical Michaelis-Menten equilibrium.

Furthermore, Verger et al. (1973) demonstrated that a striking difference between the Pancreatic phospholipase A₂ and the venom phospholipases A₂ (Bee, and Crotalus adamanteus) is the much lower induction time in an interface penetration for the latter enzymes.

In contrast with Verger et al. (1973), Wells (1974) suggested that Crotalus adamanteus phospholipase A₂ interacts with the aggregate substrate in a manner which is not fundamentally different than the manner in which it interacts with monomers. Wells (1974) proposed that the enhanced rate of hydrolysis of the aggregated substrate lies in a much lower entropy of activation. Wells suggested that the origin of this entropy difference probably arises from the fact that in the aggregated state only the reactive end of the substrate molecule is presented to the enzyme during collisions.

Very recently Roberts et al. (1977) studying the action of cobra venom (Naja-Naja) phospholipase A₂ on mixed micelles of Triton X-100 and phospholipid, proposed a "dual-phospholipid" model for phospholipase A₂ action on mixed micelles. According to this model, the enzyme must first bind calcium before it can bind phospholipid. Once calcium is bound, the enzyme binds one phospholipid molecule at the interface. This binding causes a conformational change in the enzyme which leads to dimerization. A second phospholipid is then bound by the dimer at a functional active site and catalysis occurs.

1.7. Calcium requirement of Phospholipases

Long and Penny (1957) reported the requirement of calcium and noted an inhibitory effect of zinc and copper on the phospholipase A from the Cottonmouth-moccasin venom. They suggested that the activation effect of calcium was a direct action on the enzyme because of the fact that calcium ions could not be replaced by other bivalent cations and by the inhibitory effect of ethylenediaminetetraacetic acid on phospholipase A activity.

Roholt and Schlamowitz (1961) also suggested that the effect of calcium on the phospholipase A from the Crotalus durissus terrificus venom was directly on the enzyme, and from inhibition studies with barium concluded that a metal ion-substrate complex was not involved in the reaction.

Dawson (1963) also reported that the phospholipase A from the venom of the cobra Naja-Naja requires calcium ions for activity and

noted that calcium ions cannot be replaced by adding either cetyltrimethyl-ammonium hydroxide to the lipid or magnesium ions to the buffer. With this evidence Dawson suggested that calcium ions must play a direct and specific part in the reaction between enzyme and substrate rather than merely adjusting the ζ -potential of the substrate to a favourable value. Similar conclusions have been reached by many workers.

Most recently Shipolini et al. (1971) demonstrated that the activity of Bee venom phospholipase A_2 was increased by the addition of calcium ions and the addition of EDTA abolishes the activity. The explanation of this is that the active entity is a calcium-protein complex which dissociates giving inactive protein as the calcium ions form a complex with EDTA. Calcium, which is more effective than magnesium and much more so than strontium or barium, is presumably the natural activator for the bee venom phospholipase A_2 .

Bonsen et al. (1971) and Wells (1972) have demonstrated an absolutely specific requirement for calcium using the purified enzymes from porcine pancreas and crotalus adamanteus venom respectively. Bonsen et al. (1971) demonstrated that under physiological conditions, that is to say in the assay systems containing long-chain phospholipids in the presence of bile salts, the pancreatic phospholipase A_2 is completely inactive in the absence of calcium ions. The enzyme has a high specificity for Ca^{2+} ions, the related ions Ba^{2+} and Sr^{2+} turned out to be pure competitive inhibitors for the enzyme, whereas Mg^{2+} was fully inert. Direct binding studies between the enzyme and

calcium showed that phospholipase A₂ is able to bind one metal ion per enzyme molecule. Ba²⁺ and Sr²⁺ are also bound to the enzyme (but not Mg²⁺). On the basis of kinetic data for pancreatic phospholipase A₂ obtained with micellar diheptanoyl lecithin, Bonsen et al. (1971) concluded that the addition of calcium and substrate to the porcine pancreatic enzyme was random, whereas Wells (1972) using dibutyryl lecithin in the monomeric state concluded from kinetic data for Crotalus adamanteus enzyme as well as from inhibition studies with Ba²⁺, that calcium bound to the enzyme before the addition of the substrate. Wells (1972) gave one possible explanation for these two different results. Although the venom and pancreatic enzyme catalyze the same reaction, they do so by different mechanisms. Wells (1972) proposed some evidence to support this hypothesis. The pH optimum for the pancreatic phospholipase is near 6.0 (Bonsen et al., 1971), whereas the pH optimum for the Crotalus adamanteus phospholipase is near 8.0 and it has no activity at pH 6.0. Also the pancreatic enzyme is almost inactive towards monomeric substrate whilst the venom enzyme attacks substrate in this state quite readily.

Alkaline earth cations such as Ca²⁺, Ba²⁺ and Sr²⁺ induce spectral perturbations differing for both enzymes. For the venom enzyme these were interpreted as arising primarily from the removal of a positively charged group from the vicinity of a tryptophan residue (Wells, 1973). Ethoxyformic anhydride inactivated the enzyme by acylation of one lysine (Wells, 1973). This lysine is thought to be responsible for the cation induced spectral perturbations. The spectral perturbations caused by Ca²⁺ binding to the pancreatic enzyme

were tentatively ascribed to effects on a tyrosine and a histidine residue (Pieterse et al., 1974).

The metal ion binding site consists mainly of carboxyl acid groups with an additional contribution of a more neutrally ionizing residue, presumably histidine. The idea of a histidine being involved in Ca^{2+} binding is supported by inactivation experiments with p-bromophenacylbromide. The reaction of this reagent with the active site residue His-53 is extremely retarded in the presence of calcium or substrate analogues. Conversely, when the reagent is introduced first, the modified enzyme no longer binds calcium or substrate (Volwerk et al., 1974).

In contrast to phospholipase A other phospholipases such as phospholipase B from Penicillium notatum (Dawson, 1958) and phospholipase C from Cl. perfringens (Bangham and Dawson, 1962) do not require calcium for activity. Furthermore, the activity of phospholipase B from Penicillium notatum was strongly inhibited by calcium ions (Dawson, 1958).

Phospholipase A from several mammalian tissues shows a variation in calcium requirement. Pancreatic phospholipase A_2 , as is mentioned above, and rat liver mitochondrial phospholipase A (Waite et al., 1969) require calcium ions for activity. In contrast, rat liver lysosomal phospholipase A (Franson et al., 1971) and rat intestinal mucosa phospholipase A (Epstein and Shapiro, 1959), do not require calcium for activity.

1.8 The Activation of Phospholipases by Amphipathic Compounds

Although calcium is the natural activator for the phospholipase A, there are other amphipathic compounds that can stimulate the activity of phospholipase A and other phospholipases.

The mechanism of the activation of the phospholipases by these compounds is considered to depend on the modification of the substrate by the activator to increase attraction to, or penetration of the substrate by the enzyme.

Epstein and Shapiro (1959) reported that the activity of intestinal mucosa lecithinase (phospholipase A) was dramatically increased by the addition of fatty acids, and oleic and linoleic acid were the most efficient activators. The only evidence that led Epstein and Shapiro (1959) to propose a substrate mediated mechanism for this activation, was the fact that the amount of fatty acid required for optimum activity depends upon the amount of substrate, and is roughly equimolar to it. This points to formation of a substrate-activator complex as a possible basis of activation.

However, Dawson's and his collaborators work gave the substrate-mediated mechanism theory greater support.

First, in 1958 Dawson proposed that the activation of the phospholipase B from Penicillium notatum by several lipids is due to changes of the substrate (lecithin emulsions) surface characteristics.

Bangham and Dawson (1960) studying the action of Penicillium

notatum phospholipase B on unimolecular film of lecithin reported that the activity increases with the increase of film pressure and then at a pressure of about 30 dynes/cm is sharply abolished. With high-pressure films the activity can be obtained only if negative groupings are introduced into the surface, e.g. by the addition of dicetylphosphoric acid, to form a mixed monolayer. One possible explanation is that the negative films attract a positively charged region of the large enzyme molecule and thus assist its adsorption and penetration into the film.

Phospholipase C from Cl. perfringens attacks micelle-water interfaces only when the lecithin micelle has net positive charge (ζ -potential). This was found by changing the electrophoretic mobility of the micelles by the addition of long-chain cations and anions (positive and negative charges respectively). The enzyme was active only when the micelles had a net positive charge (Bangham and Dawson, 1962).

Waite et al. (1969a,b) demonstrated that the activity of phospholipase A of rat liver mitochondria was stimulated by the addition of free fatty acids. Of various fatty acids tested, lauric, myristic, oleic and linoleic were most effective. This pattern of activation is similar to that found by Epstein and Shapiro (1959) who used phospholipase A from intestinal mucosa. Waite et al. (1969) proposed that the stimulatory effect of fatty acid resulted from increased exposure of the phospholipid to the enzyme. The evidence to support this theory was that when fatty acid was added to the phospholipase A that had been solubilized by freezing and thawing,

with the membrane fraction removed, hydrolysis was inhibited, this indicated that the stimulation by the fatty acid was not via a direct effect on the enzyme.

The first doubts that the substrate-mediated mechanism is not unique for all the phospholipases came from Dawson himself. Dawson (1963) studying the action of cobra venom (*Naja-Naja*) phospholipase A on lecithin emulsions, found no evidence of a sharp ζ -potential threshold when the enzyme is inhibited by the addition of many anionic amphipathic substances. Furthermore, the extent of the inhibition depended on the nature of the anionic amphipathic molecule and was not directly related to the ζ -potential. So Dawson (1963) concluded that the inhibition of cobra venom phospholipase A action is not so directly dependent on the ζ -potential of the substrate, in contrast with the results found with the lecithinase from Penicillium notatum and the phospholipase C of Cl. perfringens (Bangham and Dawson, 1960, 1962).

Also Verger et al. (1973) found that the previously suggested explanation that the pancreatic phospholipase A₂ requires a negative ζ -potential at the interface in order to degrade lecithin (Van Deenen et al., 1963) appears not to be true since the incorporation of short chain lecithin molecules into the organized aggregates of long chain lecithin has the same effect. Moreover, even positively charged lipid aggregates, such as lysylphosphatidylglycerol dispersions, are hydrolyzed effectively by the pancreatic enzyme.

Lawrence and Moores (1975) demonstrated activation of bee

venom phospholipase A₂ by fatty acids. The hydrolysis curve of purified egg yolk lecithin in 20% propanol assay system was biphasic with a slow early phase and a fast late phase, suggesting activation by one of the reaction products. The activation was almost entirely due to fatty acids, and lysolecithin produced much weaker activation. Conductimetric analysis showed that little interaction occurred between fatty acid and the substrate, and suggested that these compounds might be direct activators of the enzyme. Lawrence and Moores (1975) proposed an enzyme-mediated mechanism for the activation of bee venom phospholipase A₂ in contrast to the substrate-mediated mechanism. Stronger evidence to support this mechanism was obtained by studying the effect of glutaraldehyde on the enzyme activity. Incubation of the enzyme with glutaraldehyde inhibited the activated state of the enzyme but did not affect the basal activity. Experiments to determine whether the enzyme could be protected against glutaraldehyde action by oleic acid, showed that the enzyme was activated irreversibly by crosslinking in the presence of fatty acid activator. The results suggest that the crosslinking agent stabilises both low activity and high activity conformations of the enzyme, indicating that fatty acid activators cause an allosteric transition in the protein. Further evidence for the enzyme-mediated mechanism was obtained by the effect of fatty acid anhydrides on the enzyme activity. Incubation of the enzyme with long chain anhydrides activated the enzyme about 10 fold, while short chain compounds inactivated the enzyme. Furthermore, none of the acids derived from the anhydrides activated the enzyme at the same concentration as the anhydrides, and the enzyme was still

active when all the traces of activator had been removed by petroleum ether extraction. There is plenty of evidence that short chain highly reactive acylating agents inactivate the enzyme indicating that a nucleophilic residue is present at the active site. Lawrence and Moores (1975) propose that in addition a nucleophilic residue is present at an activating site and that it normally interacts with the carboxylate group of free long-chain fatty acid activators.

1.9 Temperature Optima

In comparison with other enzymes, the temperature dependence of phospholipases is unusual. Many of them show true inversion temperature or reversible loss of activity in high temperatures.

The optimum temperature of bee venom phospholipase A_2 is 65°C (Nair et al., 1976,a). Nair et al. (1976,a) reported that with egg yolk lecithin substrate the maximum activity of bee venom phospholipase A_2 was at 65°C and both dipalmitoyl and dicaproyl lecithins gave a maximum at 60°C . This loss of activity could possibly be due to the abrupt change in the physical nature of the substrate, because the enzyme is stable at very high temperatures.

Nair et al. (1976,b) investigated the influence of temperature on the activity of phospholipase A_2 present in a number of snake venoms from various species and families under the same substrate and assay conditions. Most of the venoms showed a maximum phospholipase A_2 activity at 65°C , and only a few showed optimum temperature for phospholipase A_2 activity at 55°C and 50°C . In

contrast, the crotalus venoms lost their phospholipase A₂ activity above 45°C.

A characteristic activity of bee venom phospholipase A₂ is that this enzyme has been shown to be active on a number of phosphatidyl cholines away from their respective transition temperatures. Op Den Kamp et al. (1974) reported that the bee venom phospholipase A₂ hydrolyses dimyristoyl phosphatidyl choline liposomes below, near and above the transition temperature, in contrast with the pig pancreatic phospholipase A₂ which hydrolyses lecithin liposomes only near the temperature of the gel to liquid crystalline transition.

1.10 pH Optima

The pH optimum of bee venom phospholipase A₂ is about pH 8.0 (Shipolini et al., 1971). This is in agreement with other reported pH optima from various venoms phospholipases A₂, e.g. Crotalus adamanteus venom phospholipase A₂ has pH optimum of pH 8.0 - 8.5 (Wells, 1972).

In contrast with venoms phospholipases A₂ the pH optima of phospholipases A of various mammalian tissues are different. Mitochondrial phospholipase A₂ has pH optimum above pH 8.0, while lysosomes appeared to have two phospholipases A, one with pH optimum at about pH 4.0, the second between pH 6.0 and 7.0 (Waite et al., 1969). Also pancreatic phospholipase A₂ has pH optimum between pH 5.5 and pH 6.0 (Bonsen et al., 1971).

1.11 Covalent modification of Proteins

The activity of many, perhaps the majority, of enzymes is modified by allosteric interactions with small molecules, for example, metal ions or regulatory metabolites. A large class of enzymes is also subject to modulation by covalently linked phosphate groups and the phosphorylation is ultimately under the control of cyclic AMP levels in the cell. There are very few examples in the literature where enzymic activity is regulated by covalent addition of molecular species which are closely related to free or non-covalent activators.

The only example of enzyme activation by covalent modification is that of the enzymes participating to the synthesis and breakdown of glycogen in mammals. The glycogen phosphorylase (catalyzes the breakdown of glycogen) is active when it is phosphorylated (phosphorylase "a"), otherwise it is less active (phosphorylase "b"). The phosphorylation of phosphorylase "b" is catalyzed by the activated forms of a phosphorylase kinase. Phosphorylase kinase is activated by allosteric modification with cyclic AMP (dissociation of an inactive dimer into an active subunit and a regulator subunit which binds the cyclic AMP). The active phosphorylase "a" can be attacked by another enzyme, phosphorylase phosphatase, which hydrolyzes the phosphate groups. This reaction causes phosphorylase "a" to dissociate into two half-molecules of phosphorylase "b", the less active form. The glycogen synthetase in contrast with the glycogen phosphorylase is inactive when it is phosphorylated (synthetase "D"). The phosphorylation of the active synthetase "I" (non-phosphorylated) is catalyzed by an activated synthetase kinase. The synthetase kinase is activated by cyclic AMP.

1.12 Phospholipase Assays

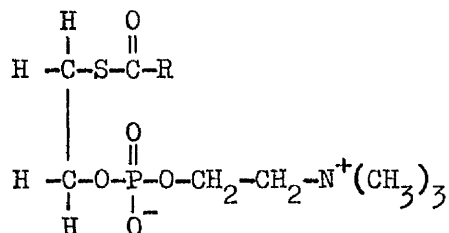
Many different methods have been introduced to follow the phospholipase activity.

The estimation of phospholipase A activity by following the decrease in acyl ester bonds occurring during the conversion of lecithin into lysolecithin, developed by Stein and Shapiro (1953), was a widely used method by early workers in the field.

Another method which still has a wide use is the detection of phospholipases activity on surface monolayers of a $[^{32}\text{P}]$ -lecithin spread on an aqueous surface, by recording the loss of surface radioactivity and the changes in the surface pressure when enzyme is introduced into the buffer (Dawson and Bangham, 1959; Bangham and Dawson, 1960).

One of the most common methods in the present years is the continuous titration of the fatty acids liberated by the action of phospholipase A on lecithins, at constant pH with a radiometer pH-stat equipment. Titration with NaOH is carried out under nitrogen.

Very recently Aarsman et al. (1976) developed a sensitive spectrophotometric assay of phospholipase A₂ and lysophospholipase. They applied acylthioester analogues of substrates such as thioglycol lecithin in measuring the activity of the phospholipase A₂ from pig pancreas and lysophospholipase II from beef liver. The advantage of this method is that the enzymes attack the acylthioester bonds several times more rapidly than that of the corresponding oxyester.



Thioglycol Lecithin[‡]

Lawrence (1971), and Lawrence and Moores (1972) applied conductimetry for assaying enzymes. This method is based on the conductance changes produced by the reaction products.

Conductimetry as a method for determining reaction rates has been known, in principle, for a considerable time (Henri and Des Bancel, 1903), but only recently has been applied to enzyme assays (Hanss and Rey, 1971; Lawrence, 1971). In the present thesis conductimetry has been used in all the enzyme assays.

The apparatus for detecting the conductance changes produced in the assay mixture, consists of the conductivity cells, a conductivity meter and a recorder. This has been developed by Lawrence (1971), and Lawrence and Moores (1972), and modified to some extent by the author. A fuller description of the above can be found in detail in the Material and Methods chapter. This method has great advantages; the apparatus is simple and requires a small volume of assay mixture, complete reaction time courses can be obtained for many reactions where previously this has not been possible. Due to the fact that the conductivity cells are small, reaction mixtures reach thermal equilibrium rapidly (within 1-2 min) and with the high

([‡]terminology by Aarsman et al., 1976)

stirring efficiency, events occurring within 10 sec may be accurately recorded. This method which provides an inexpensive assay system and is easy to use can be the ideal system for routine enzyme assays. The stability of the response allows conductance changes of 0.01%, to calibrate all this has been required modifications to the cell design and to the electronic part of the apparatus. This level of sensitivity is adequate for detailed kinetic studies of many enzyme systems. One disadvantage of conductimetry is that it is a nonspecific method, in that it cannot discriminate between different reactions. However, in the case of purified enzymes or well characterized substrates, the method gives unambiguous results. In comparison with other methods, conductivity is less sensitive than the best spectrophotometric methods, but more sensitive than titration or stopped reaction methods, but it is more general than any of these methods.

1.13 The Aims of the Present Work

The present work deals with the activation of Bee venom PLA₂ by acylating agents.

Preliminary results published by Lawrence and Moores (1975) showed that the activation of Bee venom PLA₂ by long-chain fatty acid anhydrides was due to chemical modification of the enzyme. These results set the basis for further investigation on this subject.

Many acylating agents were tested for their ability to activate the Bee venom PLA₂ and experiments were designed to elucidate the mechanism and the chemistry of the enzyme activation by these reagents. The main purpose was to find the most potent and specific activator and to establish conditions where the enzyme could be activated to very high levels and stay in this form indefinitely. The broader aims of the work were to test the activated enzyme on various substrates including cell membranes and also to determine the most probable conditions for activation of phospholipases A₂ from other sources.

Chapter II : Materials and Methods

2.1 Preparation of egg yolk Lecithin

Lecithin (1,2 acyl-3-sn-glycerophosphatidylcholine) was purified by the method of Ansell and Hawthorne (1964) based on the insolubility of the lecithin in acetone. Four dozen egg yolks were homogenized and extracted repeatedly in acetone in order to remove most of the colour. Lecithin was extracted by chloroform/methanol mixture 1/1 v/v and the solvent was then removed by evaporation. The product was dissolved in butanol and mixed with an equal volume of water in order to remove most of the ninhydrine-positive compounds which appeared on the thin-layer chromatography. The solvent phase, containing the lecithin, was dried in a rotary evaporator under vacuum and 56 g of lecithin were recovered.

2.1.1 Purification of egg yolk Lecithin

The lecithin taken from the butanol phase was not chromatographically homogenous and contained lyso-lecithin as well as other phospholipids. The product was further purified on alumina column (aluminium oxide wolen grade 1) in chloroform; lecithin was eluted with chloroform/methanol mixture 4/1 v/v and was chromatographically homogenous on thin-layer chromatography. The solvent was evaporated to dryness and the purified lecithin was dissolved in n-propanol and stored below 0°C.

2.2 Preparation of Lyso-Lecithin

Egg yolk lecithin was hydrolyzed by bee venom phospholipase A₂

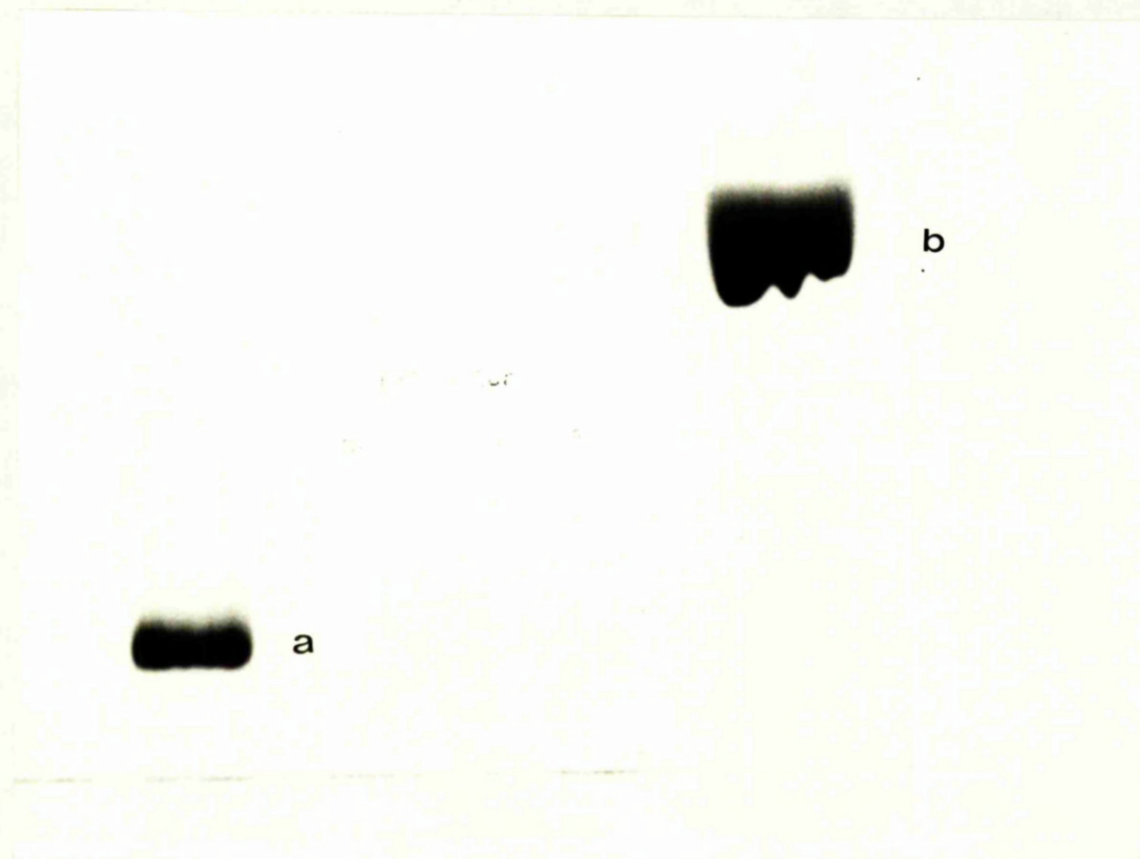


Fig (4) Thin layer chromatography of purified lysolecithin (a) and dioleoyl lecithin (b).

The chromatogram was developed with a solution containing chloroform, methanol, acetic acid and water (25:16:4:2).

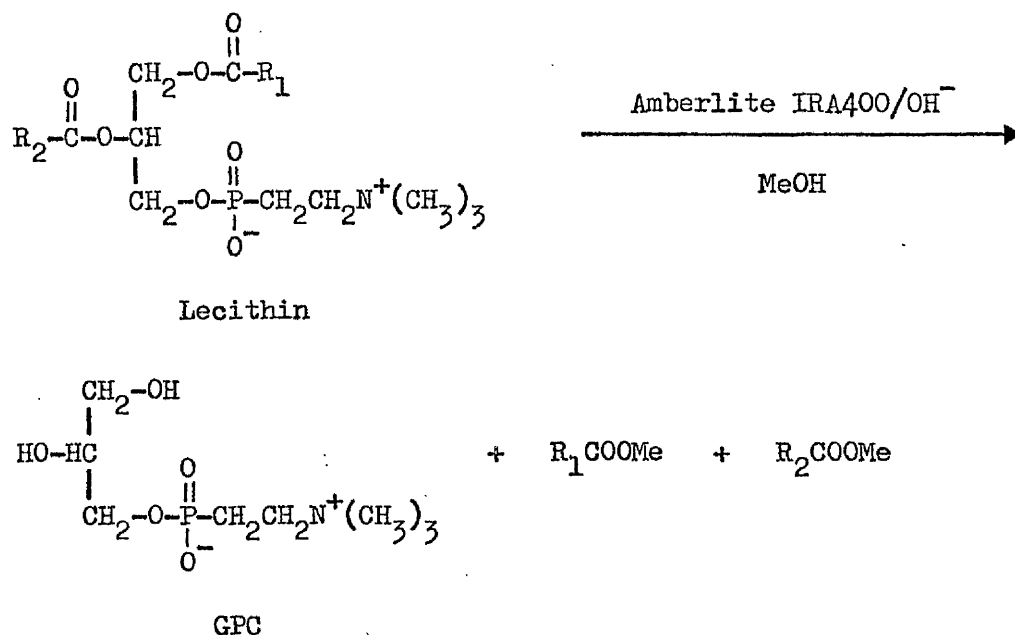
Phospholipids were stained with molybdenum blue reagent.

in order to give lysolecithin. The lecithin (100 g) was dissolved in 1000 ml n-propanol/water mixture 1/4 v/v containing 1/8 moles calcium ions and the pH was adjusted to 8.0 with NaOH. A small amount (1-2 mg) of bee venom phospholipase A₂ was added to the mixture and the reaction was followed with a pH-stat by continuous titration with NaOH. When uptake ceased lysolecithin was extracted from the n-propanol/water mixture by addition of chloroform/methanol 1/1 v/v. The organic solvent phase which contained the lysolecithin was separated and dried in a rotor evaporator. The solid was then dissolved in hot ethyl acetate and the lysolecithin precipitated on cooling leaving behind most of the impurities (Lawrence, A.J., unpublished results). The product (ca. 30 g) was further purified on an alumina column (aluminium oxide wolen grade 1) in chloroform. Lysolecithin was eluted from the column with chloroform/methanol mixture 2/1 v/v and was homogenous on thin layer chromatography (Fig. 4).

2.3 Preparation of Glycerophosphoryl Choline (GPC)

The standard method for preparing GPC, hydrolysis of lecithin using quaternary amine hydroxides in ether tended to give low yields of impure product (Brokerhof and Yurkowski, 1965). A simplified method was developed using the hydroxide form of a quaternary ammonium-based ion exchange resin to hydrolyze methanolic solutions of lecithin. This method was based on the hypothesis that hydrolysis by OH⁻ would give fatty acid anions which would remain bound to the resin while the GPC would dissolve in methanol. In practise very

little hydrolysis occurred and the fatty acids formed methanol soluble methyl esters (Lawrence and Drainas, unpublished results).



In the present study the method was modified using solvent in which GPC was insoluble while methanol esters of fatty acids remained in solution. Of the many solvents tested benzene gave the best results being noticeably superior to toluene. GPC product was formed within the resin beads, but was very readily eluted with methanol.

Experimental procedure

56 g of purified lecithin was dissolved in 250 ml benzene and stirred with 120 g of the OH⁻ form of the quaternary ammonium resin Amberlite IRA400 which had been washed in methanol and air dried. Even under these conditions sufficient methanol is present to give methanolysis rather than hydrolysis of the lecithin. 1 µl samples

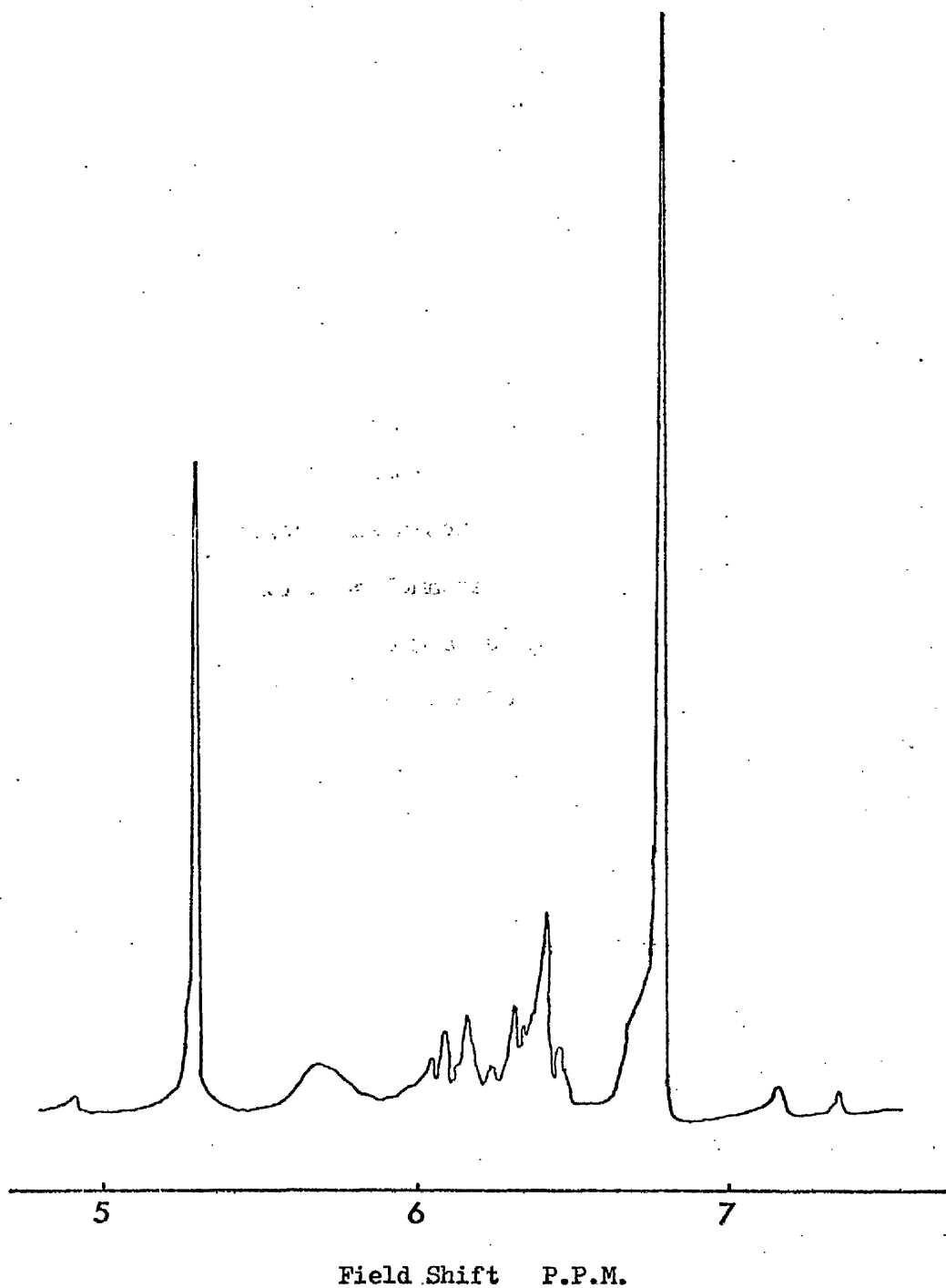


Fig (5) Nuclear magnetic resonance analysis of GPC.

The sharp peak at 6.79 p.p.m. is characteristic of protons of the $-N^+(\text{CH}_3)$ grouping.

of the solution were removed and tested for lecithin by conductimetric hydrolysis using Bee venom phospholipase A₂ and lysolecithin by thin layer chromatography. When reaction was complete, the resin was filtered and washed with benzene. GPC was eluted from the resin with methanol. The product was free from all the ninhydrin positive compounds which remained on the resin after the methanol wash and were recovered by washing the resin in a solution of 10% acetic acid prepared in methanol. The methanol solution of GPC was dried in a rotary evaporator under vacuum and GPC was further dried in a vacuum dessicator in the presence of phosphorus pentoxide.

A single element analysis gave the following data: Analysis calculated for C₈H₂₀O₆PN (MW, 257):

C, 37.5; H, 7.78; O, 37.35; N, 5.45; P, 12.06. Found:

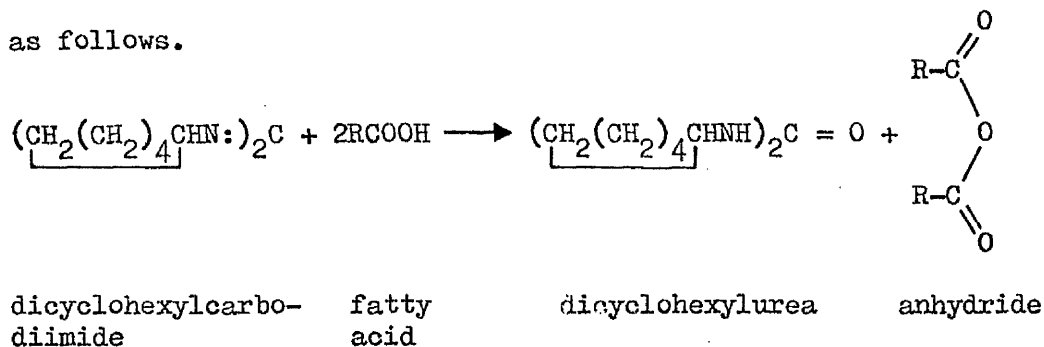
C, 36.63; H, 7.70; O, - N, 5.53; P 10.4 . The product was dissolved in CD₃OD and characterized by NMR. This gave a sharp peak for the protons of the -N⁺(CH₃)₃ grouping and a complex peak for the other protons with approximately equal peak areas (Fig. 5).

This method of GPC preparation is essentially a one step procedure and avoids many purification steps in contrast with many other methods. Also it gives very good yields of GPC, 12 g of GPC out of 56 g of lecithin (ca. 75% of the theoretical).

2.4 The preparation of fatty acid Anhydrides

Anhydrides were prepared by the method of Selinger and Lapidot (1966). A two molar excess of the fatty acid was mixed with

dicyclohexylcarbodiimide in petroleum ether. The reaction proceeds as follows.

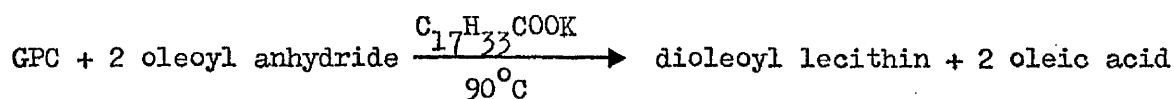


The dicyclohexylurea derivative which is insoluble in petroleum ether was removed by filtration and the petroleum ether was removed by evaporation.

2.5 The synthesis of di-acyl-glycerophosphatidylcholines

2.5.1 The synthesis of Dioleoyl Lecithin

Dioleoyl lecithin was synthesized from GPC and oleoyl anhydride in the presence of potassium oleate.



GPC, oleoyl anhydride and potassium oleate were mixed in a proportion of 1/4/0.2 w/w/w respectively.

Due to the fact that GPC does not dissolve in long chain fatty acid anhydrides, the mixture was stirred vigorously and heated at a high temperature ($> 100^\circ\text{C}$) in an oil bath. This helped GPC to dissolve and to speed up the reaction which would normally be very slow. When GPC was totally dissolved, temperature was kept constant at 90°C .

Short chain fatty acid anhydrides normally dissolve GPC, so the reaction is therefore easier and faster.

All reactions were followed by thin-layer-chromatography.

The product was taken up with petroleum ether which leaves behind the potassium salt, dried in a rotary evaporator and purified on alumina column (aluminium oxide wolen grade 1) in chloroform. Dioleoyl lecithin was eluted with chloroform/methanol mixture 4/1 v/v, and deionized with a mixed-bed ion exchange resin Zerolite D.M.F. The product was homogenous on thin-layer chromatography (Fig. 4). The dried dioleoyl lecithin was dissolved in n-propanol and stored below 0°C.

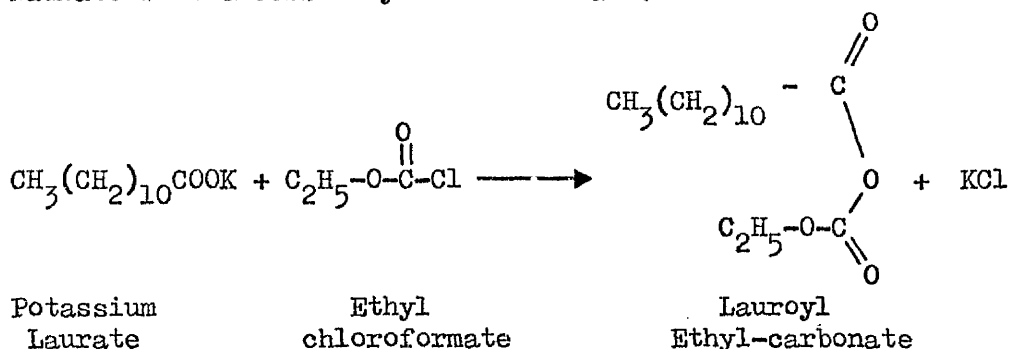
Dihexanoyl, dioctanoyl, didecanoyl, diundecenoyl lecithins were synthesized by this method.

2.6 The Synthesis of 2-acyl derivatives of egg yolk Lysolecithin

2-decanoyl and 2-lauroyl derivatives of egg yolk lysolecithin were synthesized from lysolecithin and fatty acid anhydride in the presence of the potassium salt of the fatty acid in proportion 1/2/0.2 w/w/w respectively. The reaction was carried out at 90°C and followed by thin layer chromatography. The product was taken up with petroleum ether, dried in a rotary evaporator and purified on alumina column (aluminium oxide wolen grade 1) in chloroform. The 2-acyl derivative was eluted with chloroform/methanol mixture 4/1 v/v and deionized with a mixed bed ion exchange resin Zerolite DMF. The product was homogenous on thin layer chromatography.

2.7 The preparation of Ethyl-Carbonate derivatives

Lauroyl ethyl carbonate was prepared by mixing the potassium laurate with excess ethyl chloroformate.

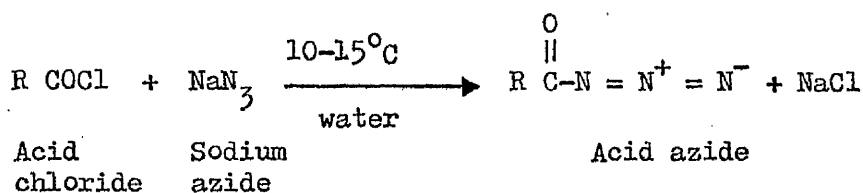


The excess ethyl chloroformate was removed by evaporation in a rotary evaporator and the product was taken up with petroleum ether, dried and dissolved in ether as 2% w/v stock solution. The product lauroyl ethyl carbonate was characterized by infrared analysis. Oleoyl ethyl carbonate was prepared by the same method.

2.8 The preparation of Fatty acid Azides

Lauroyl and oleoyl azides were prepared from the acid chlorides and sodium azide by two methods:

a) As is described by Fieser and Fieser (1967). A mixture of acid chloride and acetone was added from a dropping funnel with cooling and stirring to a solution of sodium azide in water at a low temperature (10-15°C). After the reaction was completed (ca. 1 h) the organic phase was washed several times with cold water.



b) A solution of acid chloride in acetone was passed a few times

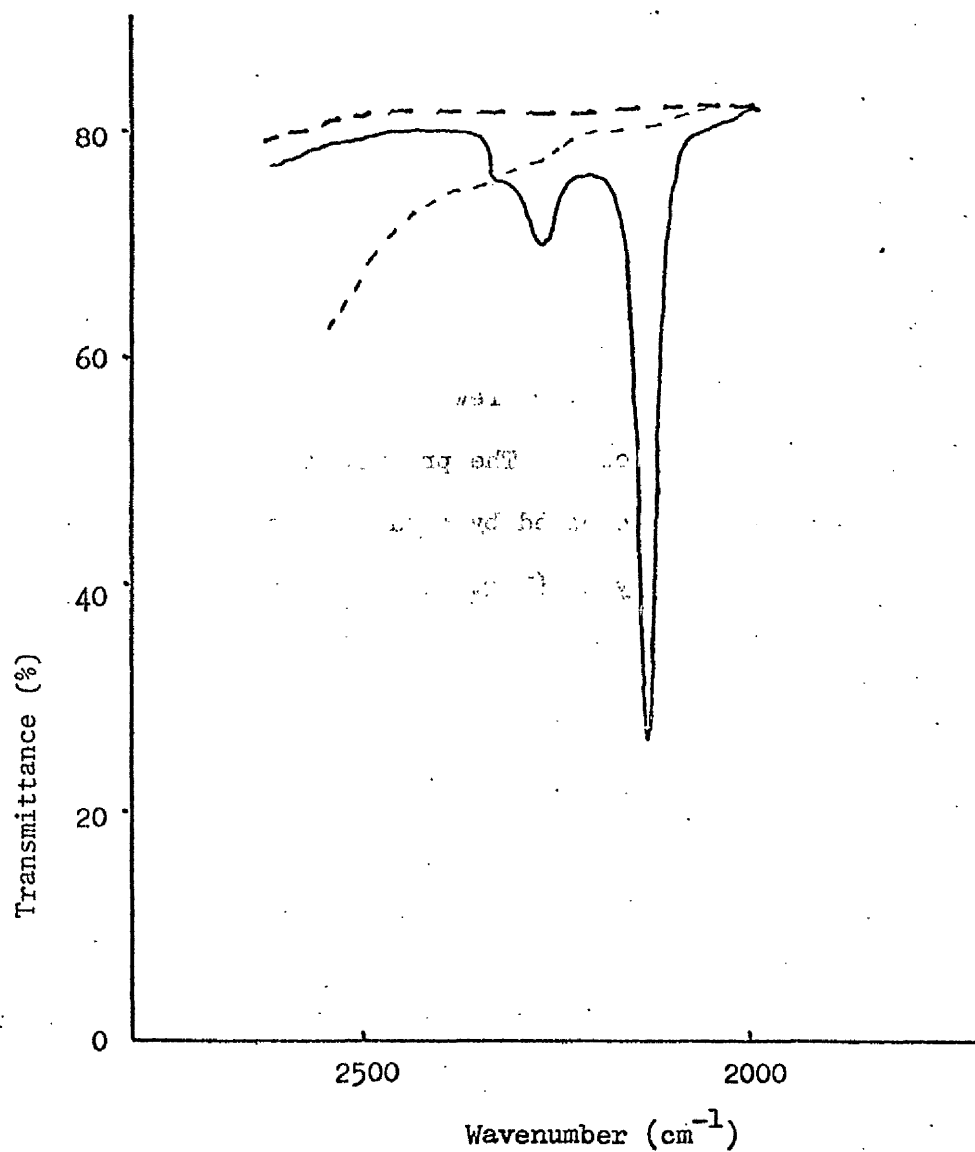


Fig (6) Infrared analysis of Lauroyl azide.

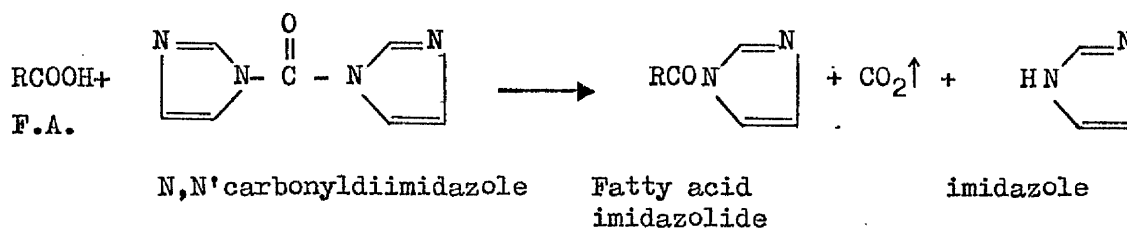
The figure shows the characteristic peak of the acid azide which is not present in the acid (-----) or acid chloride (-----).

through a column of acetone washed resin amberlite IRA 400 equilibrated with azide ion (Lawrence, A.J., unpublished results). 50 ml of solution was treated with 40 g of resin and each passage took ca. 1 min. Conversion was followed by conductimetry and was complete in four passes. The azide product was vacuum dried for infrared analysis and used within a few hours of synthesis. This method is far more satisfactory. The product was free of acid chloride and lauric acid as checked by conductimetry and characterized as an azide by infrared analysis (Fig. 6).

2.9 The preparation of Fatty acid Imidazolides

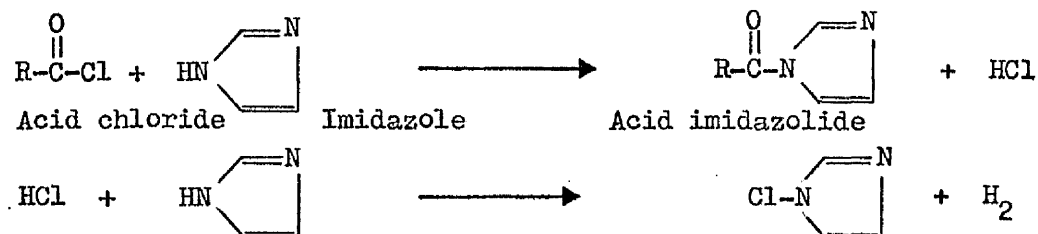
These compounds were prepared by two methods.

- a) Fatty acid and N,N'-carbonyldiimidazole were mixed in equimolar quantities in dry acetone at a final concentration of 100 mM.



This method was used to make small quantities of imidazole derivatives of a large range of fatty acids.

- b) Oleoyl and lauroyl imidazolides were also prepared by treating the acid chlorides with a two molar excess imidazole in toluene solution (Lawrence, A.J., unpublished results).



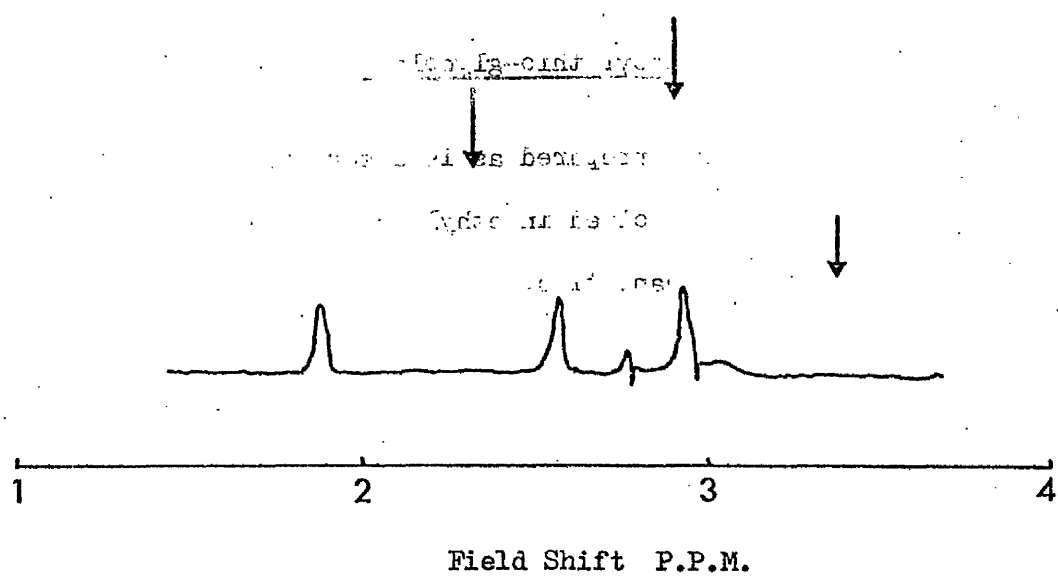


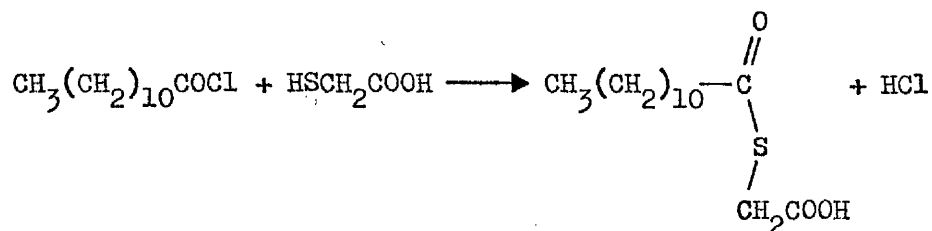
Fig (7) Nuclear magnetic resonance analysis of Oleoyl imidazolidine.

The arrows show the peak positions for pure imidazole.

The solvent phase was separated and evaporated to dryness and the solid extracted with petroleum ether which was then removed by evaporation. The products were free of all traces of fatty acid, imidazole and acid chloride (as checked by conductimetric analysis) and characterized by NMR (Fig. 7).

2.10 Preparation of Lauroyl thio-glycolate

This reagent was prepared as is described by Albertson (1962). Thioglycolic acid was dissolved in ethyl acetate and lauroyl chloride was added in equimolar quantities.



After three to four hours at room temperature the mixture was washed with water and concentrated to give the thiol ester.

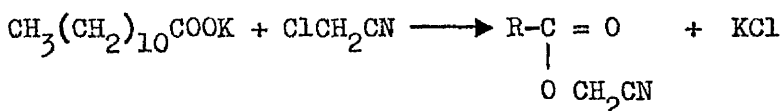
2.11 Preparation of Lauroyl 1 cyclohexyl-3(2-Morpholinoethyl) carbodiimide

This reagent was prepared by mixing equimolar quantities of lauric acid and 1 cyclohexyl-3(2-Morpholinoethyl) carbodiimide in acetone.

2.12 Preparation of Lauroyl acetonitrile

This reagent was prepared as is described by Albertson (1962).

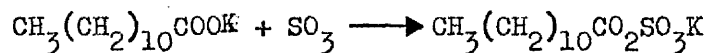
Potassium laurate and chloroacetonitrile were mixed in equimolar quantities.



Potassium laurate	Chloro- acetonitrile	lauroyl acetonitrile
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2.13 Preparation of Lauroyl sulfate

Lauroyl sulfate was prepared by adding sulfur trioxide in the form of its crystalline dimethylformamide complex to the alkali metal salt of lauric acid in dimethylformamide.



The reaction of the sulfur trioxide-dimethylformamide complex with the potassium laurate in dimethylformamide is complete within 1 minute at 0°C.

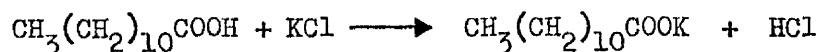
2.14 Radioactive Reagents

2.14.1 $[^{14}\text{C}]$ -Lauroyl ethyl carbonate

The potassium salt of $[^{14}\text{C}]$ -lauric acid was mixed with purified ethyl chloroformate in order to give $[^{14}\text{C}]$ -lauroyl-ethyl carbonate.

250 μCi of $[^{14}\text{C}]$ -lauric acid of specific activity 32 $\mu\text{Ci}/\mu\text{Mol}$ and total weight 15.6×10^{-4} g were mixed with 133.06×10^{-4} g of

cold lauric acid. This mixture was dissolved in petroleum ether and equimolar quantity of KCl was added in order to synthesize the potassium salt of the acid.



The product was dried in a rotary evaporator under high vacuum and excess ethyl chloroformate was added, following the same procedure as is described above, to form ^{14}C -lauroyl-ethyl-carbonate. The product was dissolved in diethyl ether at a final concentration of 2% w/v solution. The specific radioactivity of ^{14}C -lauroyl-ethyl carbonate was 3.3 $\mu\text{Ci}/\mu\text{Mol}$.

2.14.2 ^{14}C -Oleoyl imidazolid

Equimolar quantities of ^{14}C -Oleic acid mixed with cold oleic acid and N,N'-carbonyldiimidazole were mixed in dry acetone to form ^{14}C -oleoyl imidazolid at a final concentration of 100 mM, giving ten fold dilution of the initial radioactivity. The reaction between oleic acid and N,N'-carbonyldiimidazole is described above. The specific radioactivity of ^{14}C -oleoyl imidazolid was 0.21 $\mu\text{Ci}/\mu\text{Mol}$.

2.15 Purification of crude bee venom PLA₂ by Gel filtration

Purified bee venom PLA₂ was a gift from Dr. R. Shipolini (University College London).

The crude bee venom PLA was purified by repeated passage through a column of Bio Gel P-30 (Laurence, A.J., unpublished results). A small sample of crude bee venom PLA₂ was applied on a Bio Gel P-30

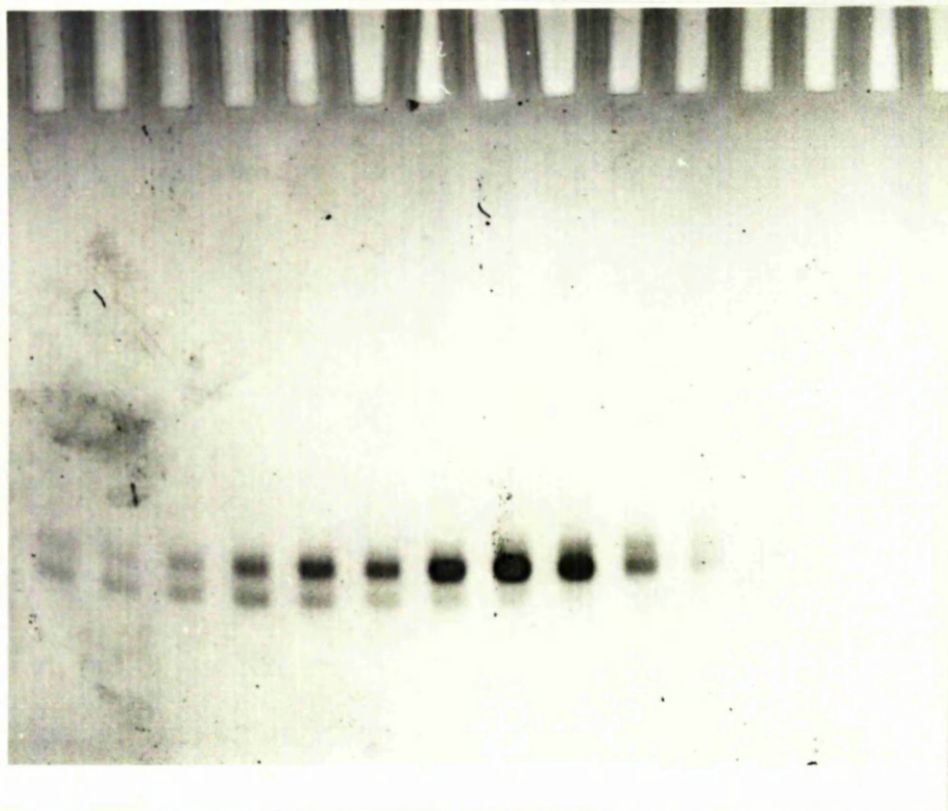


Fig (8) Gel electrophoresis of the Bee venom PLA₂ fractions derived from a Bio Gel P-30 column.

column preswollen in NaCl solution 0.1M at 0°C. The purity of the column fractions was checked by acrylamide gel electrophoresis, and the procedure was stopped when the PLA₂ fractions gave a single band on the acrylamide gel (Fig. 8).

Gel electrophoresis was run using an acid/urea system (Riggs et al., 1977) with 14% acrylamide monomer containing 0.3% bis acrylamide made in 5% acetic acid in the presence of 3M urea and run with 5% acetic acid in both electrodes.

2.16 Gel Electrophoresis

The isoelectric focussing system was made by using 3-10, 7-10 or 9-11 5% ampholines prepared in 7% acrylamide, 0.3% bis acrylamide solution in total volume of 40 ml. The acrylamide was set with 90 µl of 10% fresh prepared ammonium persulfate solution and 20 µl TEMED. The tank buffers were; 2% acetic acid solution for the anode and 1.25% ethanolamine solution for the cathode and the protein samples were run under constant voltage (ca. 300 V) for at least 10 hours. The protein bands on the gel were precipitated with 12.5% (w/v) trichloroacetic acid solution (Stoddart and Price, 1976). Cytochrome C and myoglobin were used as marker proteins and were visible throughout the run.

The cathode-running gel system was made with 14% acrylamide, 0.3% bis acrylamide prepared in a 3M urea, 5% acetic acid solution in total volume of 40 ml, the acrylamide was set with 200 µl of

10% fresh prepared ammonium persulfate solution and 50 μ l TEMED. The tank buffer for both electrodes was 5% acetic acid (Riggs et al., 1977). Samples were run under constant current (30 mA) for 3 hours. The proteins were stained with 0.2% coomassie blue solution containing 50% methanol and 7% acetic acid and the gel was destained in a 7% acetic acid; 5% methanol solution.

2.17 Enzyme Assays

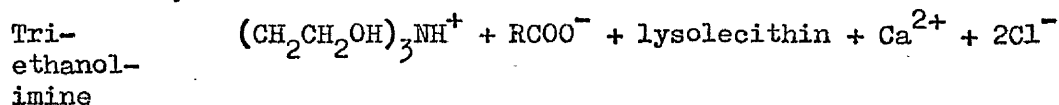
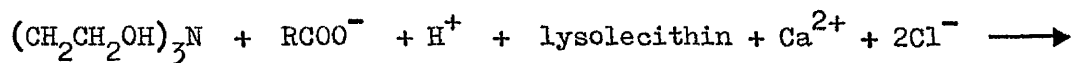
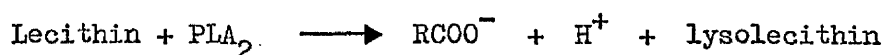
Bee venom phospholipase A₂ activity was assayed following the release of fatty acids from the 2-position of 1,2 acyl-3-sn-glycero-phosphatidylcholine. Reactions were followed by conductimetry using one of the following reaction mixtures.

- a. n-propanol/water, 1/4 v/v, buffered at pH 8.2 with 10 mM triethanolamine - HCl containing 0.1 mM CaCl₂.
- b. Water, pH 8.2, containing 10 mM triethanolamine - HCl buffer with or without 0.1 mM CaCl₂.
- c. Water, pH 8.2 containing 10 mM bicine ; NaOH buffer with 1 mM Ca²⁺.

Enzyme assay was based on the fact that hydrolysis of an ester gives an acid anion and a proton. The proton is absorbed by the buffer species with one of two results. a) Increase in the net positive charge and hence increase in buffer species mobility, or b) decrease in the net negative charge and hence decrease in buffer species mobility. The former occurs with cationic buffer species (e.g. tris,

triethanolamine) and the latter with anionic buffer species (e.g. phosphate, bicine, MOPS). Hence the use of cationic buffers gives a very sensitive assay while use of anionic buffers does not. However there are circumstances in which the use of cationic buffers fails. Very long chain acid residues form non-conducting ion-pairs in aqueous solution. This tendency is reversed by addition of organic solvents particularly n-propanol. If the reaction is carried out at high calcium concentration the released fatty acid preferentially binds calcium and gives a large reduction in solution conductance. This is enhanced by protonation of an anionic buffer.

Fatty acids with acyl chains longer than twelve carbons interact with calcium ions in water at concentrations encountered in this system. However, it has been found that in propanol/water 1/4 v/v system there is no detectable ionic interaction under the experimental conditions. The reaction product fatty acid and lysolecithin react with the buffer (propanol/water, triethanolamine) as follows:



Therefore at the end there is a gain of two independently mobile ions in solution ($(\text{CH}_2\text{CH}_2\text{OH})_3\text{NH}^+$ and RCOO^-) which give a continuous increase to the conductance. The linearity of conductance change as a function of the release of fatty acid anion to the reaction mixture has been checked by the addition of free fatty acids to the reaction mixture in

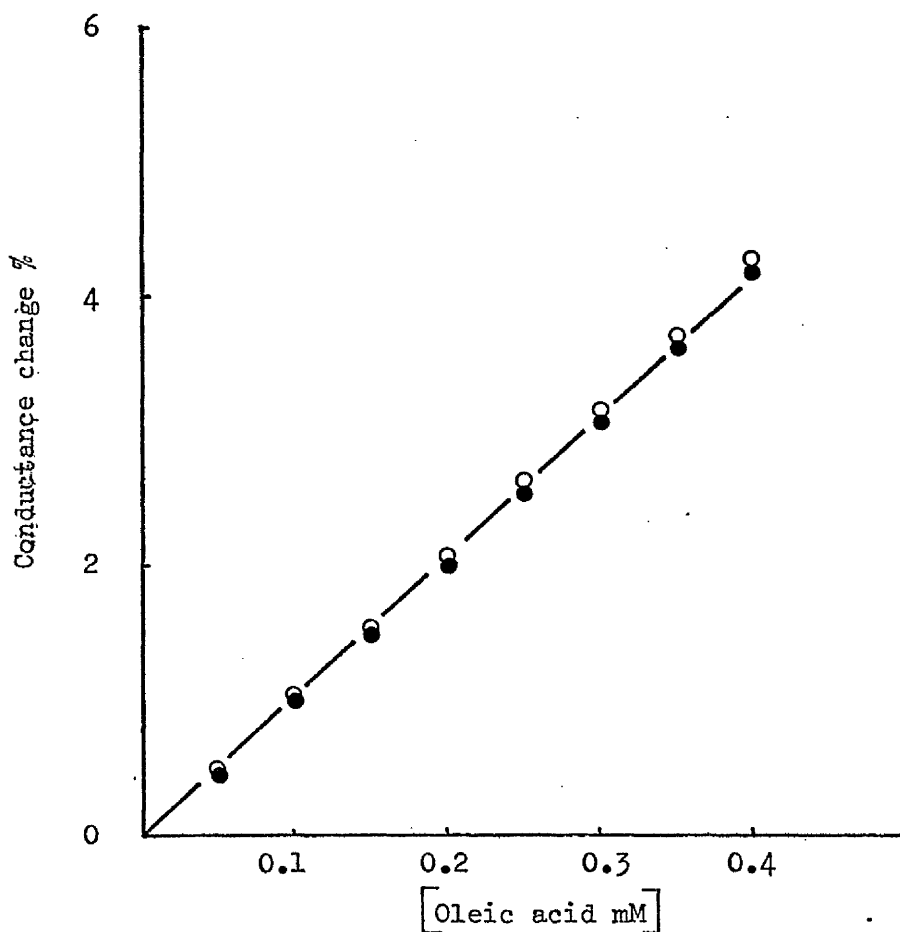


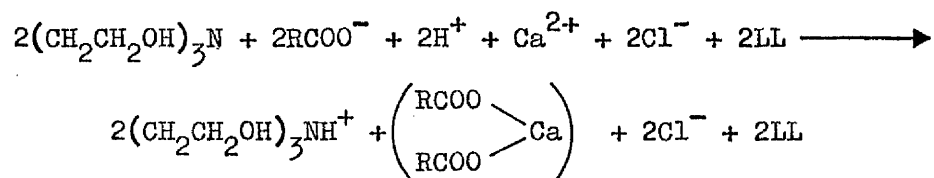
Fig (9) Calibration of the 20% propanol system with oleic acid.

The oleic acid (100 mM solution in n-propanol) was added to, 1 ml 20% n-propanol-water v/v, 10 mM triethanolamine/HCl pH 8.2 containing 0.1 mM CaCl_2 solution set at 37°C , in the presence or absence of dioleoyl lecithin.

- without dioleoyl lecithin
- with 0.24 mM dioleoyl lecithin

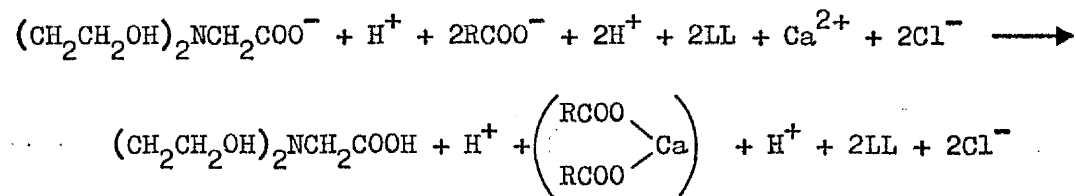
the presence or absence of the substrate and was found that the conductance changes were linear function of the fatty acid additions (Fig. 9).

The water-triethanolamine buffer system can be used only for short acyl chain lecithins ($R \leq C_{12}$) and follows the same type of reaction mentioned above. The hydrolysis products of long acyl chain lecithin ($R > C_{12}$) react with this buffer system as follows:



Therefore at the end there is no significant change in the total ion mobility of the reaction mixture, giving as a result no detectable conductance changes.

The water-bicine (N,N-bis(2-hydroxyethyl)glycine) buffer system has been used to follow reactions with long acyl chain lecithins in water ($R > C_{12}$), recording the Ca^{2+} precipitation. The reaction products react with the buffer (bicine) as follows.



Therefore at the end there is a loss of two conducting ion charges which give a continuous decrease of the conductance of the reaction mixture. The conductance changes in this system have been checked by the addition of long acyl chain fatty acids to the reaction mixture and

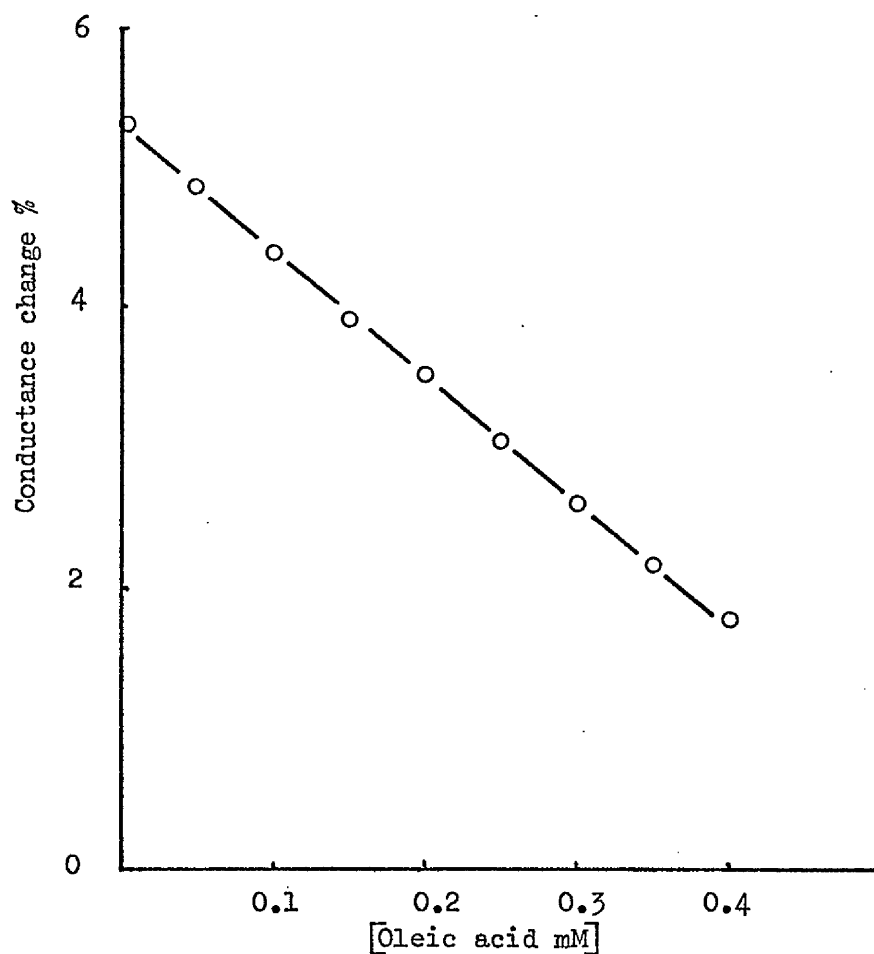


Fig (10) Calibration of the aqueous-Bicine system with oleic acid.

The oleic acid (100 mM solution in n-propanol) was added to 1 ml 10 mM Bicine/NaOH, pH 8.2 containing 1.0 mM CaCl_2 solution, set at 37°C .

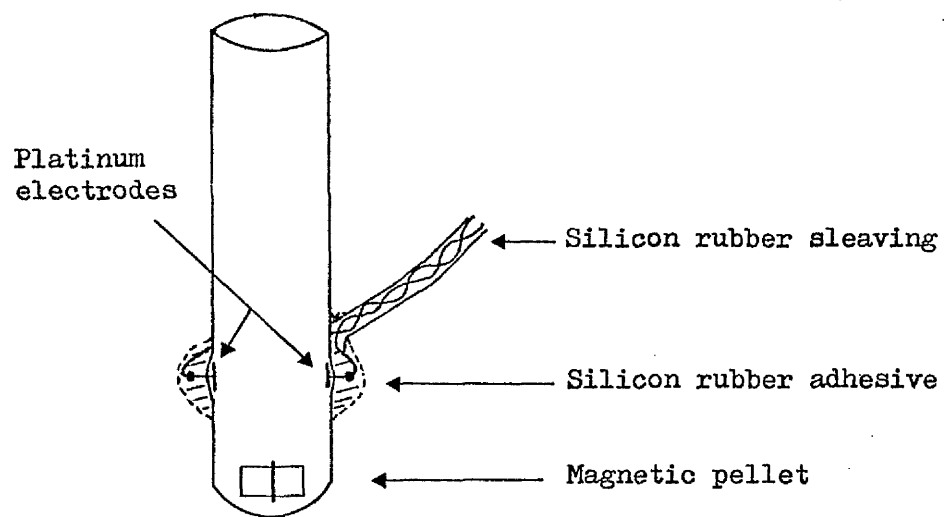


Fig (11) The conductivity cell

it was found that the decrease of the conductance was a linear function of the fatty acid additions (Fig. 10).

2.18 Apparatus

2.18.1 Conductivity Cells

Lawrence et al. (1974) described a conductivity cell suitable for kinetic studies. This cell suffered from the disadvantage that the electrode system was fragile and the cell constant could be altered by mechanical damage so that recalibration was frequently required. A new type of cell was designed according to the following principle. The cell is a small glass tube, 50 mm long and 8 mm diameter, with the electrode leads passing through two small holes in the side of the glass and resealed by melting the glass and further sealed with silicone based adhesive (Fig. 11).

It is known that at low applied voltage and frequency solutions obey Ohm's Law. Resistance varies directly with the distance " ℓ " between the two electrodes in the conductivity cell and inversely with the area of each " a ".

$$R = p \frac{\ell}{a} \text{ ohm's } (1)$$

where " p " is a constant, the "specific resistance."

For a given cell " ℓ " and " a " are constant and the quantity ℓ/a is called the cell constant " K ".

$$K = \frac{\ell}{a} (2)$$

The "Specific conductance" designated by "k", of a given solution is defined as $1/\rho$ ohm's $^{-1}\text{cm}^{-1}$ hence (1) may be written

$$R = \frac{\ell}{ka} = \frac{K}{k} \quad (3)$$

If the conductance of the solution is represented by "C", then

$$C = \frac{1}{R} = \frac{ka}{\ell} = \frac{k}{K} \text{ ohm's}^{-1} \text{ or mhos} \quad (4)$$

The platinum electrodes were cleaned by routine methods and were not platinised. The use of low frequency AC eliminates polarization problems at the electrodes.

The conductivity cells are placed in a glass water jacket connected to a thermostat with very good temperature control. The solution is stirred continuously by placing a small magnetic pellet inside the conductivity cell and a magnet stirrer underneath. Mixing and thermal equilibration are both rapid and readings are unaffected by changes in sample volume above a nominal capacity which could be as low as 0.6 ml.

The distance between the electrodes and the bottom of the cell is critical, because under a minimum length the stirring interferes with the response of the cells. So the electrodes are placed at a minimum height of 12 mm. Furthermore the response is sensitive to interference due to the formation of small bubbles on the electrodes and this can be prevented by degassing the warmed reaction solution at a vacuum pump on the day of use.

The electrode leads and the wires leading to the conductivity

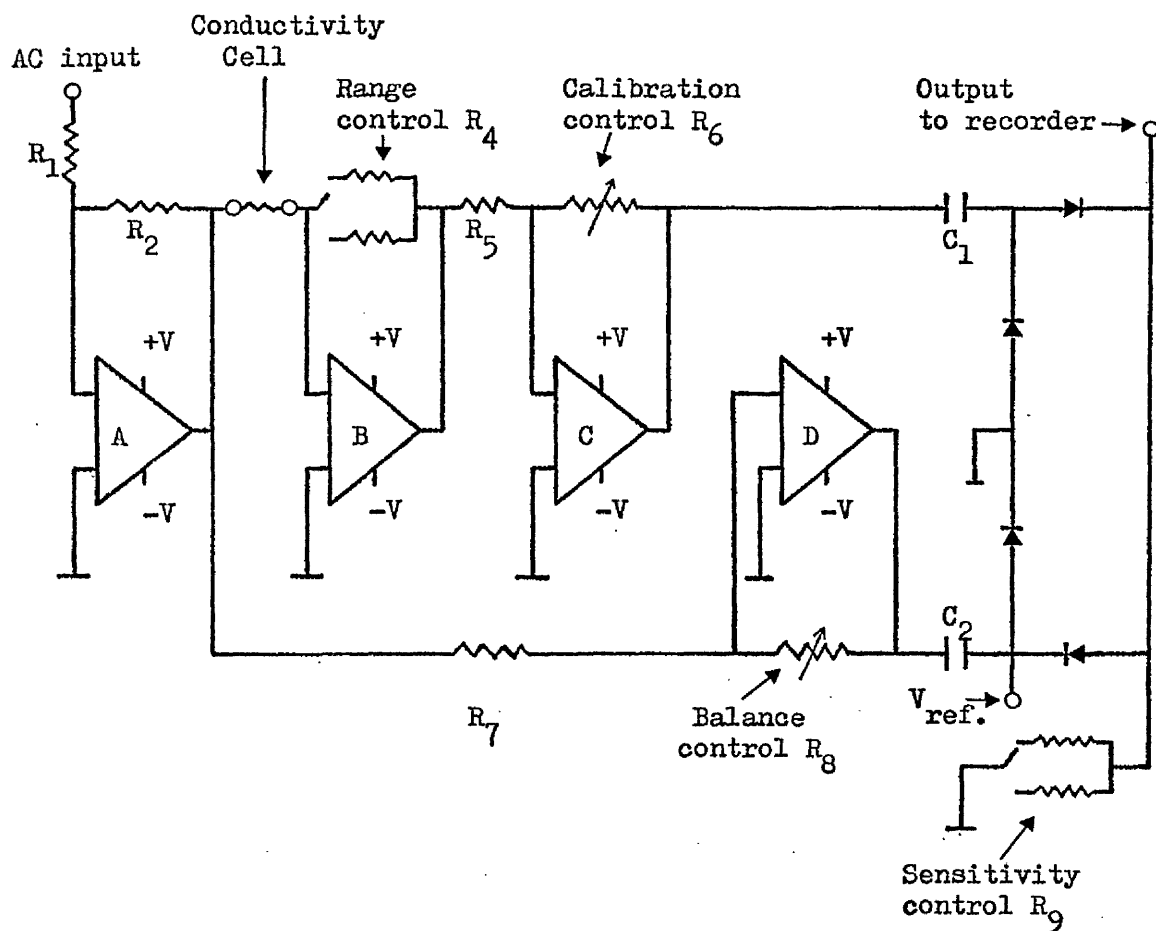


Fig (12) The modified conductivity bridge circuit.

The component values for the bridge circuit are :

R_1, R_2, R_5 $100K\Omega$, R_7 , $10K\Omega$, R_4 values are chosen from the series $1M\Omega$, $330K\Omega$, $100K\Omega$, $33K\Omega$, sensitivity is attenuated by using resistors in the series 5, 10, 20, 40, 80 Ω , R_6, R_8 $100K\Omega$ variable, C_1, C_2 $22nF$, diodes 0A47, operational amplifier 741 type (A,B,C,D).

When the internal voltmeter calibration is used voltages are matched at the point $V_{ref.}$ and C_2 values for different meters are matched better than $\pm 0.05\%$.

meter are covered with silicone rubber adhesive, a water resistant insulator which has made this design possible.

The major improvement is that the electrode leads are very well insulated and passed through the thermostat bath rather than back into the cell. The electrode surface is very firmly embedded in the glass wall of the tube and cell constants are extremely stable. In a given batch of cells made to the same specification cell constants tended to vary by less than $\pm 20\%$.

The conductimetric technique was not investigated in detail, but the accuracy and significance of results was verified by routine internal calibration for reproducibility and linearity. Thus all reaction solutions were calibrated by addition of aliquots of fatty acid both in the presence and absence of phospholipids.

The conductivity bridge system applied a constant voltage AC signal to the cells and no significant differences in response were noticed for variation in applied voltage (in the range 0.5-2 volts peak to peak) or in frequency (in the range 2000 - 3000 Hz).

2.18.2 The Conductivity Meter

The six-channel conductivity meter was essentially as described by Lawrence et al. (1974), but some important modifications were introduced, which affected the convenience of operation of the instrument.

Conductance is measured with a low-frequency AC bridge (Fig. 12)

in which an amplitude stable signal from a modified multivibrator is fed via a common input stage (A) into a linear conductance amplifier (B + C) and a reference amplifier (D).

A rectifying network performs an algebraic subtraction of the conductance - determined and reference signals to give a DC output of 0-100 mV.

In the six-channel system a stabilised power supply (-15,0, 15 V at 100 mA) and oscillator supply six bridge units and the outputs are recorded by a multipoint or a two-point recorder of 1 mV input sensitivity.

Each stage of the bridge is an operational amplifier in the inverting mode with an input resistor R_{in} and feedback resistor R_f ; voltage gain is R_f/R_{in} , hence response is a linear measure of input conductance or feedback resistance and output impedance is very low.

The output of stages (B + C) is therefore a linear measure of cell conductance and of stage (D) is a linear function of the resistance of R_7 , the balance control, which is calibrated 0-1000 Units.

Off-balance output is a direct measure of cell conductance change, with stage (C) to compensate for differences in sensitivity due to differing cell constants.

As is mentioned above, cell constant "K" is proportional to the distance "ℓ" between the two electrodes and inversely proportional

to the area of each, "a". When constructing small conductivity cells, it is very difficult to keep the parameters "Q" and "a" constant. Very small variations give different cell constants for each conductivity cell and therefore a correction is required to watch the sensitivities of the cells in a multichannel instrument.

The instrumental correction for differences in cell constants which allowed all cells to respond at the same sensitivity was originally made by a preset alignment of an internal and an external control for the reference voltages. Each alignment for the cell variation could only be made to approximately 1%. In the modified system the instrument incorporated an internal voltmeter for measuring reference voltages on each channel. Thus preset calibration of each balance control was avoided. The external control was then used to obtain zero voltage output when all cells (containing a standard solution) were balanced against the same reference voltage (Lawrence, A.J. and Drainas, D., unpublished results).

All channels thus give the same scale deflection on the chart paper for a given change in ion concentration (regardless of the initial solution conductance).

Chapter III The Action of Bee venom Phospholipase A₂
on Lecithin

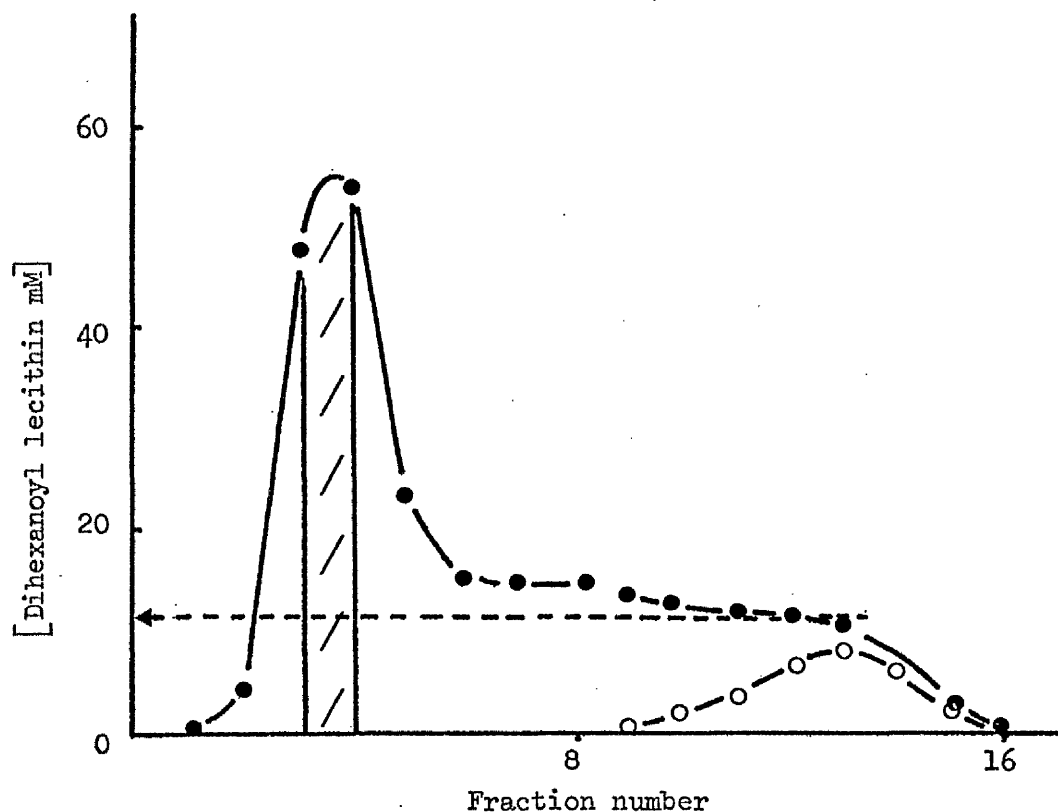


Fig (13) Determination of the critical micelle concentration of dihexanoyl lecithin.

A column (1.5 x 20 cm) of Sephadex was equilibrated in aqueous assay buffer at 25°C and 0.5 ml of dihexanoyl lecithin (470 mM in water) applied and 0.7 ml fractions collected. ●. Phospholipid concentration was determined by conductimetric assay (comparing the total hydrolysis of the samples by PLA_2 with a known concentration of hexanoic acid). A fraction from the excluded peak (marked) was re-chromatographed. ○. The CMC was estimated from the plateau concentration.

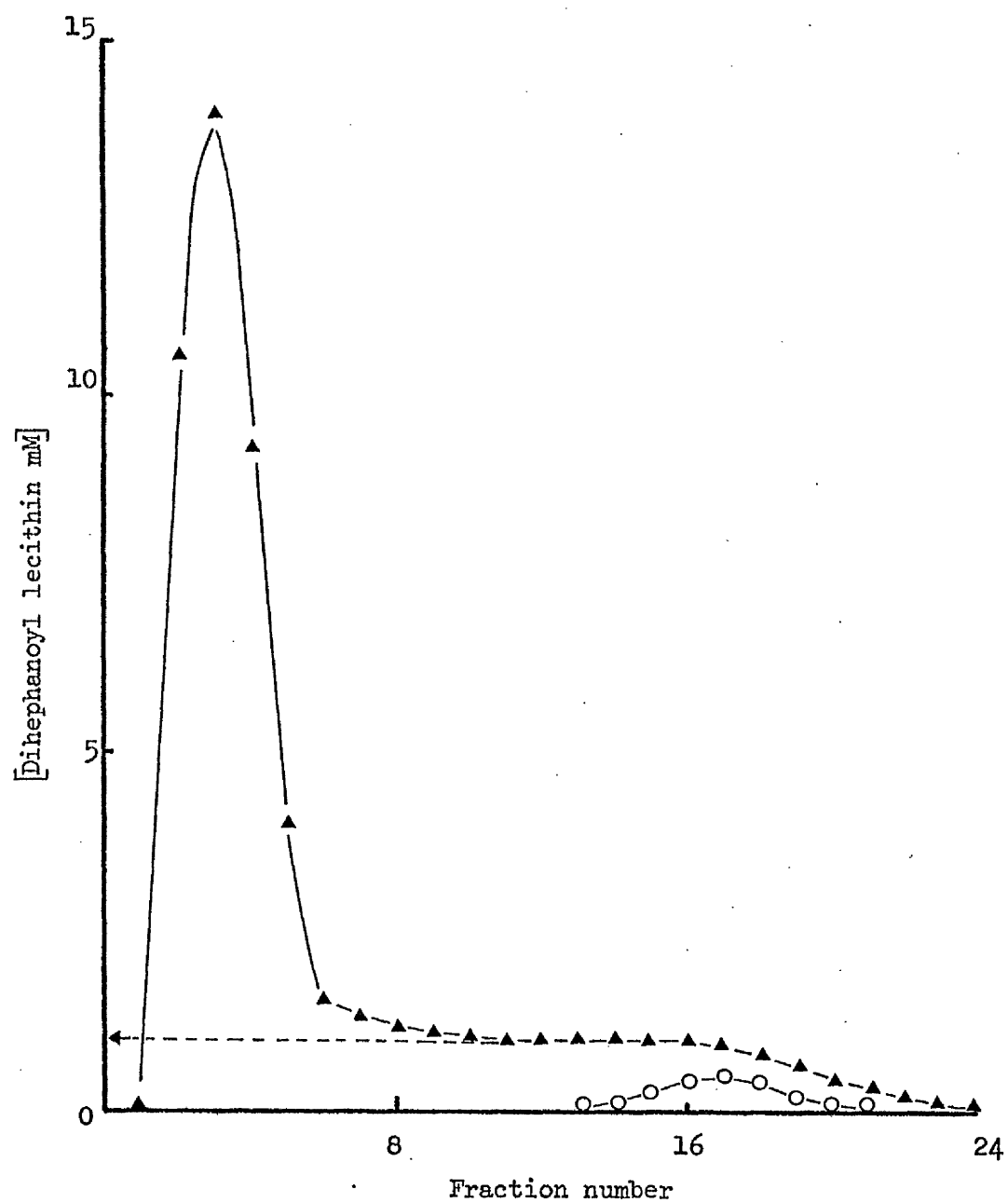


Fig (14) Determination of the CMC for diheptanoyl lecithin.

The same procedure as in Fig 13, but at 37°C.

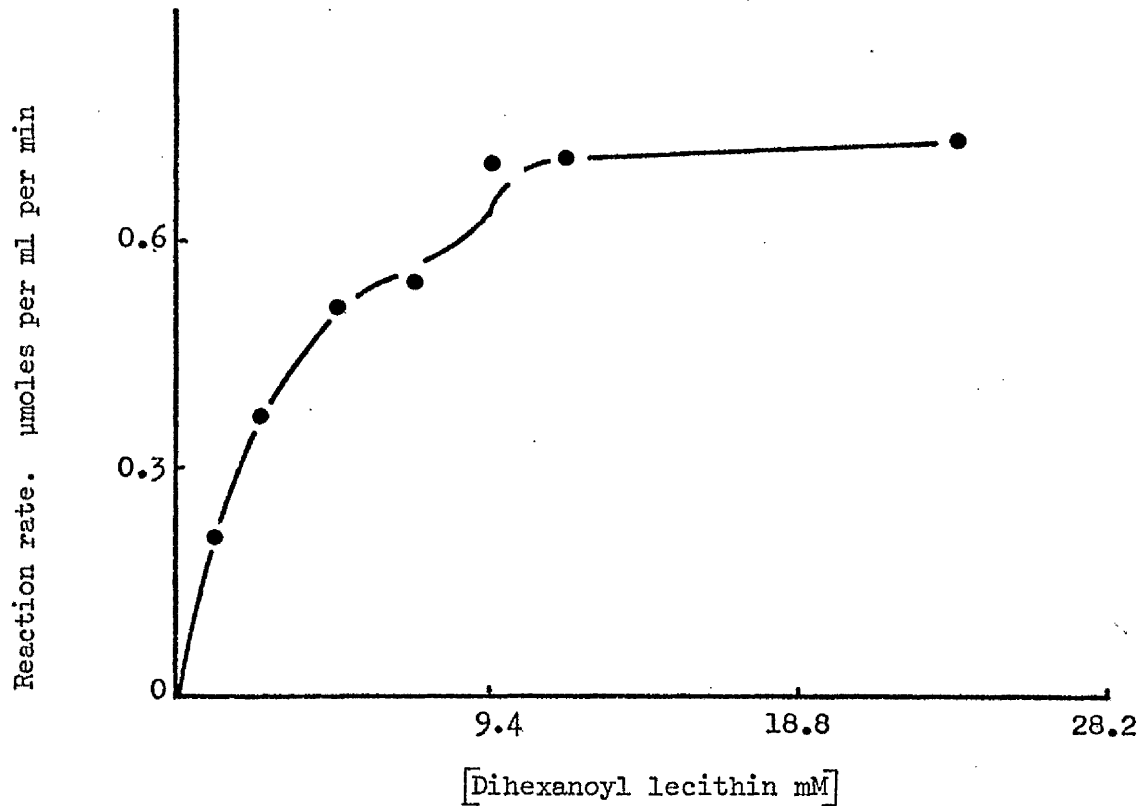


Fig (15) The initial rates of hydrolysis of dihexanoyl lecithin by bee venom PLA₂ in aqueous assay buffer (10 mM triethanolamine/HCl pH 8.2 containing 0.1 mM CaCl₂) at 25°C.

Two kinds of assay systems have been used during the course of the present work, the 20% propanol assay system and the aqueous assay system (see Materials and Methods chapter). The action of bee venom phospholipase A_2 towards lecithin is different in these two systems due to the substrate structure, and will be discussed for both short acyl-chain ($R \leq C_{12}$) and long acyl-chain lecithins ($R > C_{12}$).

3.1. The action of Bee venom PLA₂ on short acyl-chain lecithins
($R \leq C_{12}$)

Lecithins with both acyl chains shorter than C_{12} form pure micelles above a critical concentration in water, the critical micelle concentration (CMC). The structure of these lecithins in 20% propanol system is not clearly defined, they may exist either as monomers or as expanded micelles at any concentration.

The susceptibility of monomer and micellar substrates to the bee venom enzyme was examined in a concentration range spanning the CMC and in no case was a definite change of the behaviour of the enzyme clearly observed to occur at the CMC (Shipolini et al, 1971).

Short chain symmetrical lecithins were studied by gel filtration (Fig 13, 14) and by hydrolysis with porcine pancreatic phospholipase A_2 to examine the nature of the monomer/micelle transition (Bonsen et al, 1971) and to obtain values for the critical micelle concentrations. The results with dihexanoyl lecithin (Fig 15) do not confirm earlier studies (Shipolini et al, 1971) as the micellar form of diheptanoyl lecithin does appear to be more susceptible than

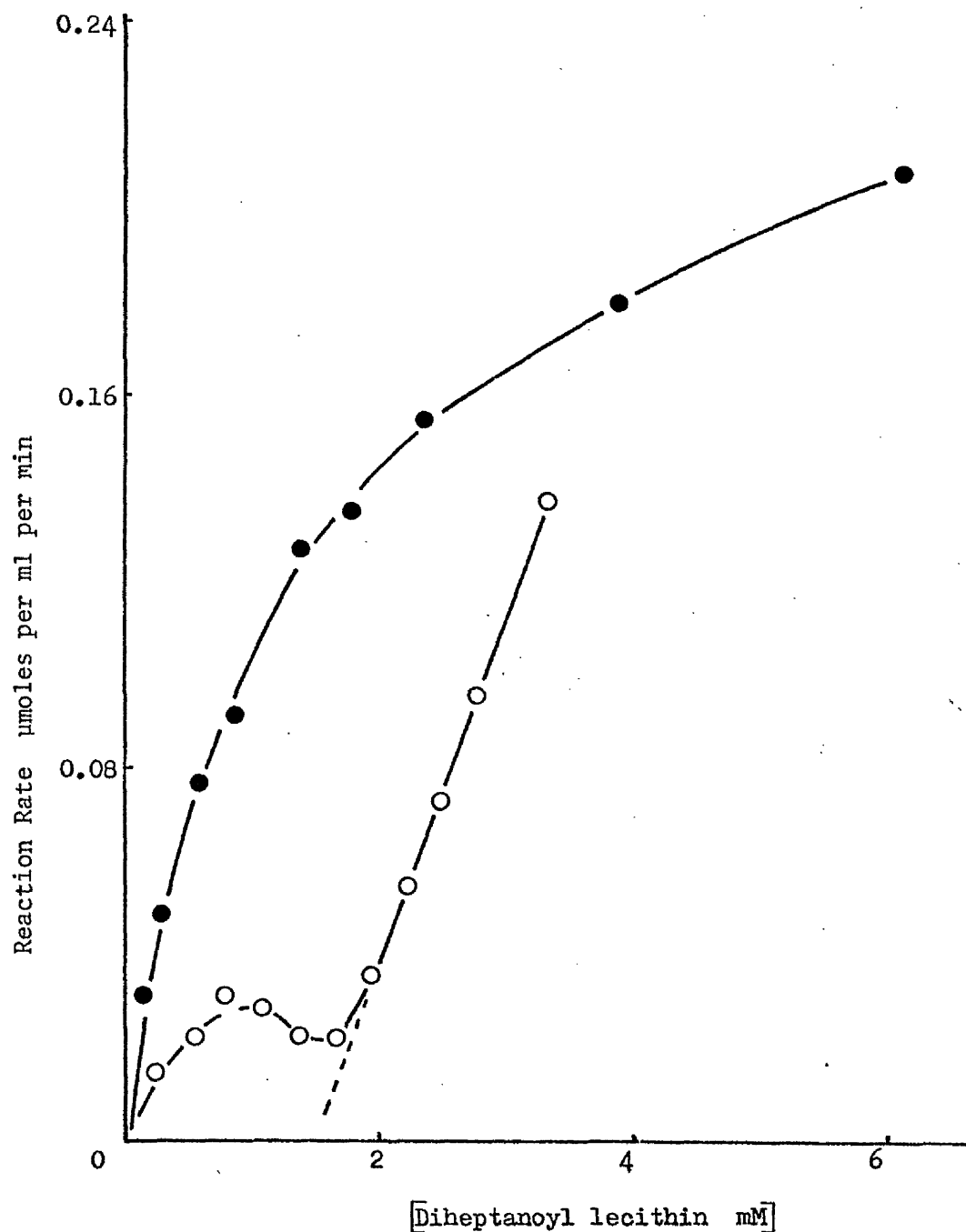


Fig (16) The susceptibility of diheptanoyl lecithin to porcine pancreatic \bigcirc and bee venom \bullet phospholipases A_2 in aqueous assay buffer (10mM triethanolamine/HCl pH 8.2 containing 0.1 mM $CaCl_2$) at $37^\circ C$. The CMC determined by the extrapolation shown was 1.4mM .

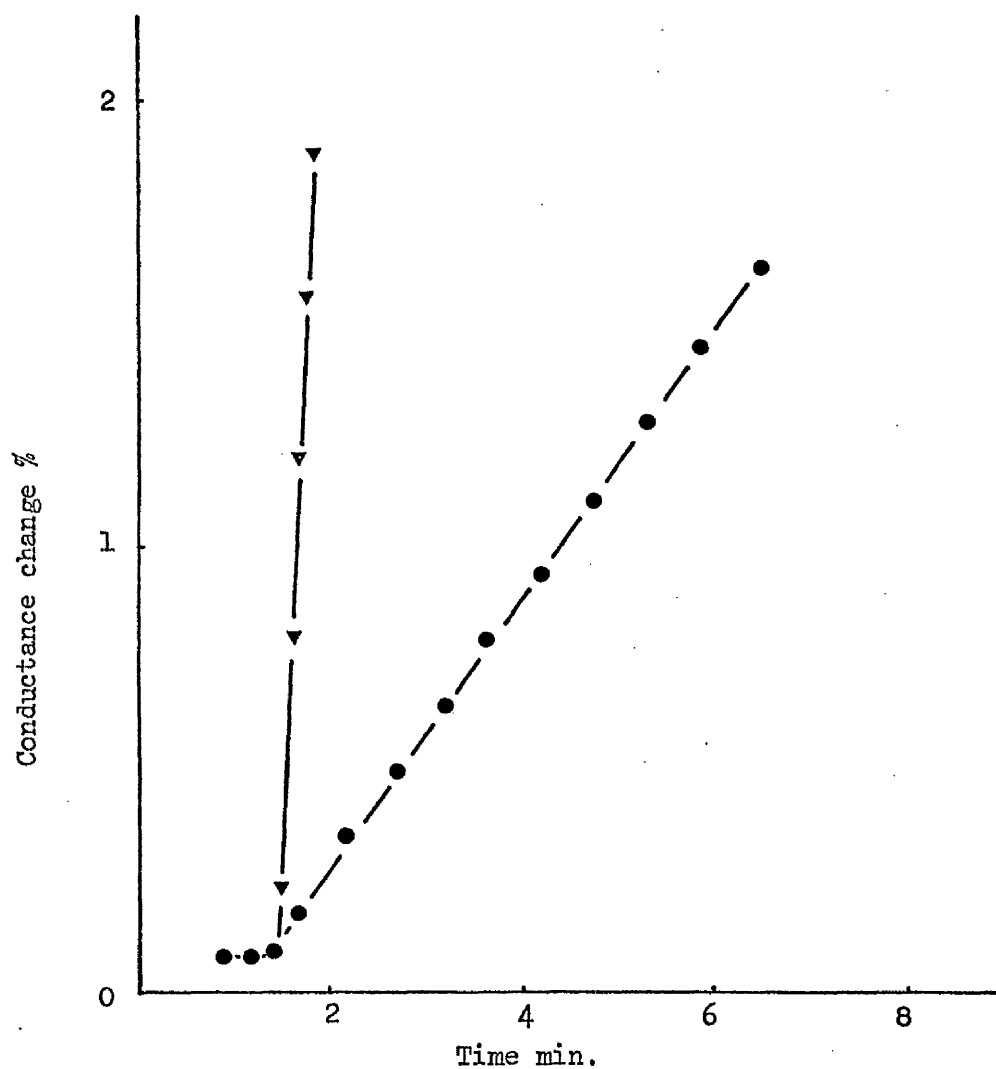


Fig (17) The hydrolysis of 2-hexanoyl lecithin by bee venom PLA₂ in aqueous and in 20% propanol system at 37°C.

- 20% propanol-water v/v, 10 mM triethanolamine/HCl, pH 8.2 containing 0.1 mM CaCl₂.
- ▼ 10 mM triethanolamine/HCl, pH 8.2 containing 0.1 mM CaCl₂.

the monomer (Fig 16). Dioctanoyl lecithin saturates the enzyme at much lower concentrations and rate changes cannot be sufficiently well resolved to support precise statement about the CMC. As the monomer concentration may increase above the critical value (de Haas et al, 1971), this kind of test is not very satisfactory for the bee venom enzyme, but the results suggest that the micellar form in any case is not much more susceptible than the free monomer for the short chain compounds.

Fig 17 shows the action of bee venom PLA₂ towards short chain symmetrical lecithins in aqueous and in 20% propanol system. It is clear that PLA₂ attacks these lecithins rapidly in aqueous solution, in contrast with the hydrolysis of the same lecithins in 20% propanol solution which is rather slow. Therefore it is probable that the intrinsic catalytic activity of the enzyme is lower in media containing organic solvents.

3.2. The action of Bee venom PLA₂ on long acyl-chain lecithins

Lecithin with both acyl chains longer than C₁₂ form liposomes in water. The structure of these lecithins in 20% propanol system is not defined; they appear as particles which cannot readily be characterized as liposomes.

The bee venom PLA₂ attacks long-acyl chain phosphatidylcholine derivatives most rapidly in dilute aqueous solutions of organic solvents (e.g. 20% propanol), in contrast to its action on short chain derivatives. This may be simply due to increased solubility of the

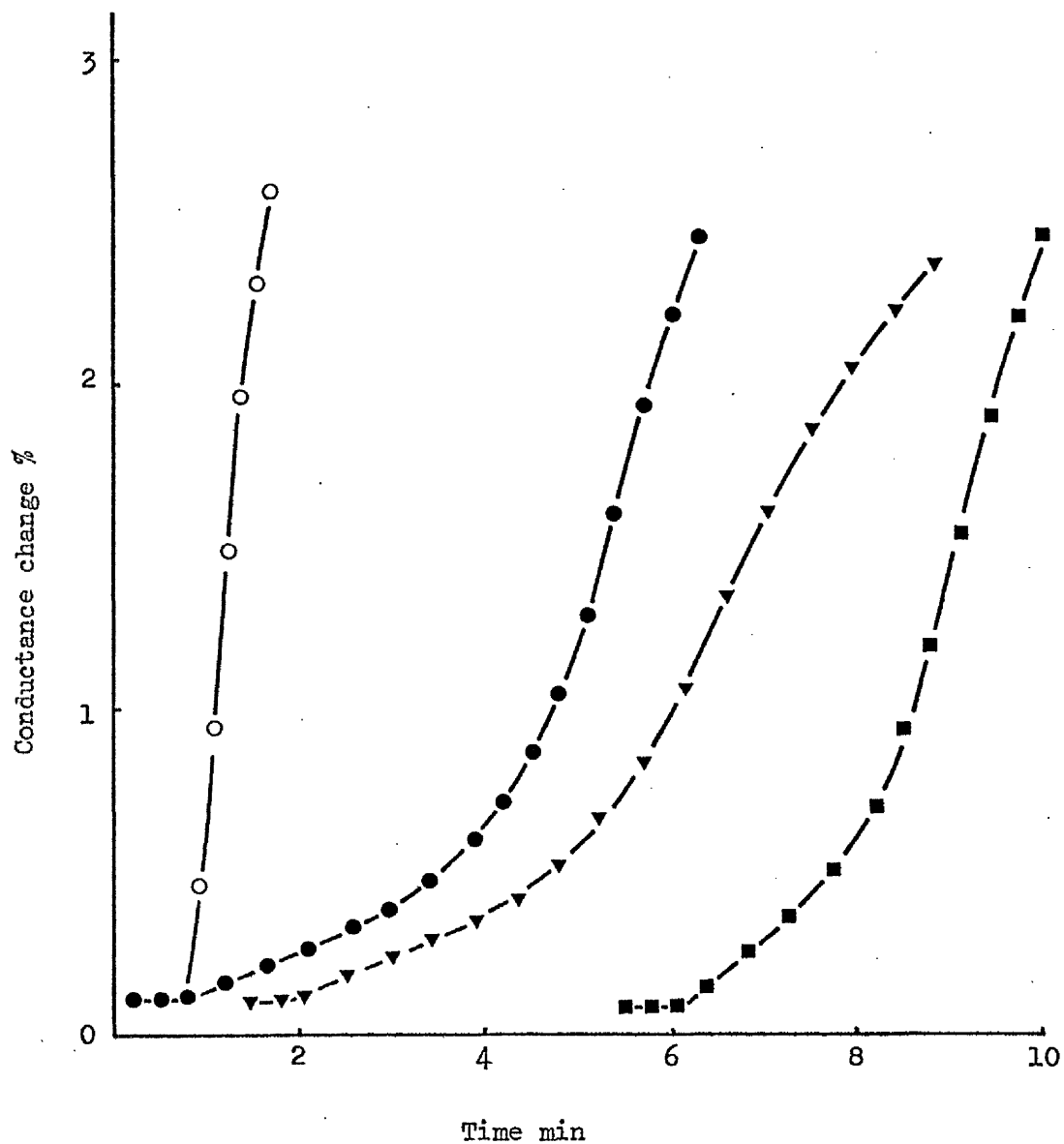


Fig (18) The hydrolysis of dioleoyl lecithin (0.24 mM) by bee venom PLA₂ (2.5 μg/ml) in the 20% propanol assay system at 37°C.

Dioleoyl lecithin was added to 1 ml 20% propanol-water v/v, 10 mM triethanolamine/HCl pH 8.2 containing 0.1 mM CaCl₂.

●-● Control, ■-■ with 0.03 mM lysolecithin,
 ○-○ with 0.15 mM oleic acid, ▼-▼ with 1 μl
 of triton X-100 solution (10% w/w in water).

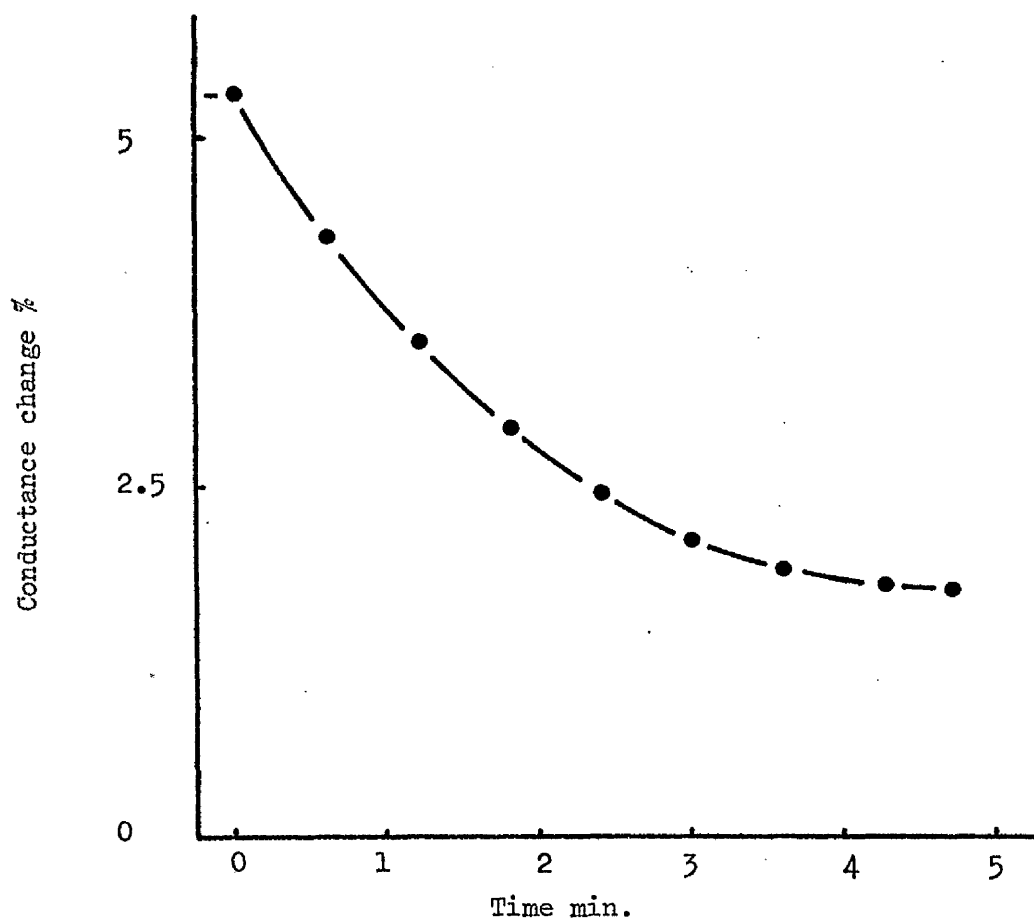


Fig (19) The hydrolysis of Dioleoyl lecithin by Bee venom PLA₂ in the aqueous assay system.

Dioleoyl lecithin (0.48 mM) was added to 1 ml 10 mM Bicine/NaOH, pH 8.2 containing 1 mM CaCl₂ and the reaction was carried out at 37°C with 1 µg per ml enzyme.

long chain lecithins in the presence of organic solvent. Under optimum conditions for the hydrolysis of egg yolk lecithin and dioleoyl lecithin, in the 20% propanol system, the reaction is biphasic with a slow early and fast late phase (Fig 18). This is due to product activation by fatty acids and to a much smaller extent to lysolecithin. Fig 18 shows the stimulation of the PLA_2 action on dioleoyl lecithin by the addition of free oleic acid and the weaker effect of free lysolecithin and the detergent Triton X-100.

Natural lecithin contains mainly long-chain phospholipids, so the study of phospholipase action towards these phospholipids is very important. Dioleoyl lecithin shows the same behaviour to the enzyme activity as natural lecithin in both assay systems (20% propanol and aqueous). This compound is easy to make (see Materials and Methods) and is soluble under the experimental conditions, and in view of the necessity to use a well characterized long-chain substrate to study the action of bee venom PLA_2 , it forms an ideal substrate.

Although conductivity could be used in studying the action of bee venom enzyme towards long-chain lecithins in the 20% propanol/water-triethanolamine system, difficulties arose with the aqueous-triethanolamine system, because long chain fatty acids bind calcium very strongly under these conditions. To overcome this problem and to be able to record the enzyme activity by conductimetry under these conditions the reaction was followed at high calcium concentration in the presence of an anionic buffer (Bicine), and the resultant fall in conductance was due to the calcium precipitation and to the protonation of the buffer by the released fatty acids (Fig 19).

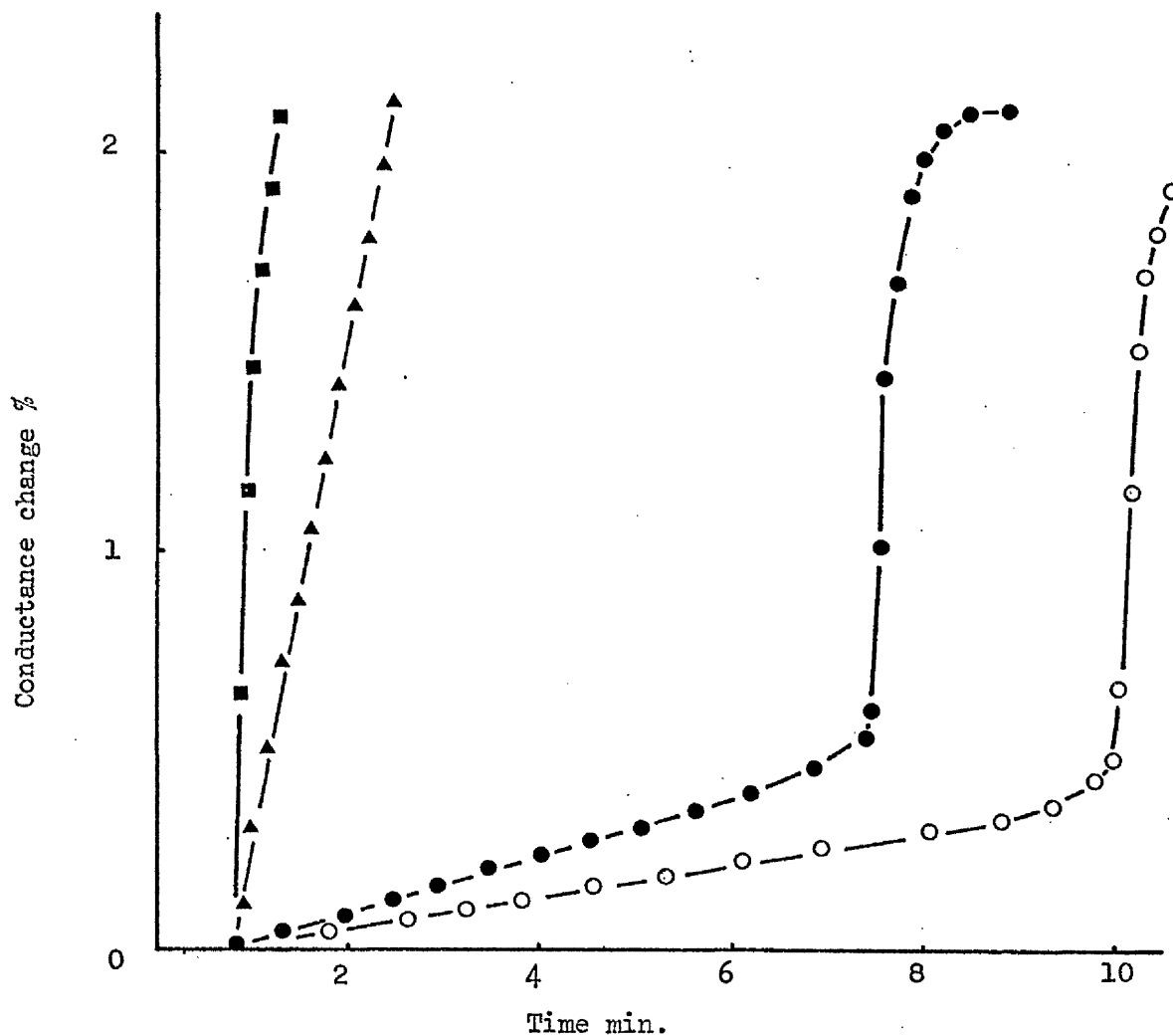


Fig (20) The hydrolysis of 2-lauroyl lecithin (0.1 mM) by bee venom PLA₂ (4 µg per ml) in the aqueous triethanolamine assay system at 37°C.

●-● Control, ■-■ with 1 µl of triton X-100 solution (10% w/w in water), ▲-▲ with 0.04 mM lysolecithin, ○-○ with 0.015 mM lauric acid.

3.3. The action of Bee venom PLA₂ on 2-acyl derivatives of purified egg yolk lecithin

The 2-acyl derivatives of purified egg yolk lecithin with $R_2 > C_9$ form liposomes in water. In 1-position egg yolk lecithin has palmitic (61%), stearic (25%) and the rest mainly oleic acid (Menzel and Olcott, 1964).

The bee venom PLA₂ attacks these liposomal substrates in the water-triethanolamine system much more slowly than micellar substrates. The reaction is biphasic with a long initial phase, which is so slow that no reaction rates can be measured with any confidence; this is followed by a very fast late phase (Fig 20). This rapid acceleration of reaction rates is due to product activation. Tests with fatty acid and lysolecithin products showed that this activation was entirely due to the latter compound. The released lysolecithin disrupts the liposomes to form micelles on which the enzyme has much greater activity (Roholt and Schlamovitz, 1961). This type of activation can be demonstrated by addition of free lysolecithin or detergents in the reaction mixture. Fig 20 shows that the addition of free lysolecithin or triton X-100 abolishes the lag phase of the reaction, and also shows that free fatty acids have very little effect. This product activation will be called a non-ionic detergent type, in contrast with the product activation in the 20% propanol system as described above. 2-acyl derivatives of egg yolk lecithin with $R_2 < C_9$ do not show product activation.

Chapter IV : The Activation of Bee venom PLA₂ in
20% propanol system

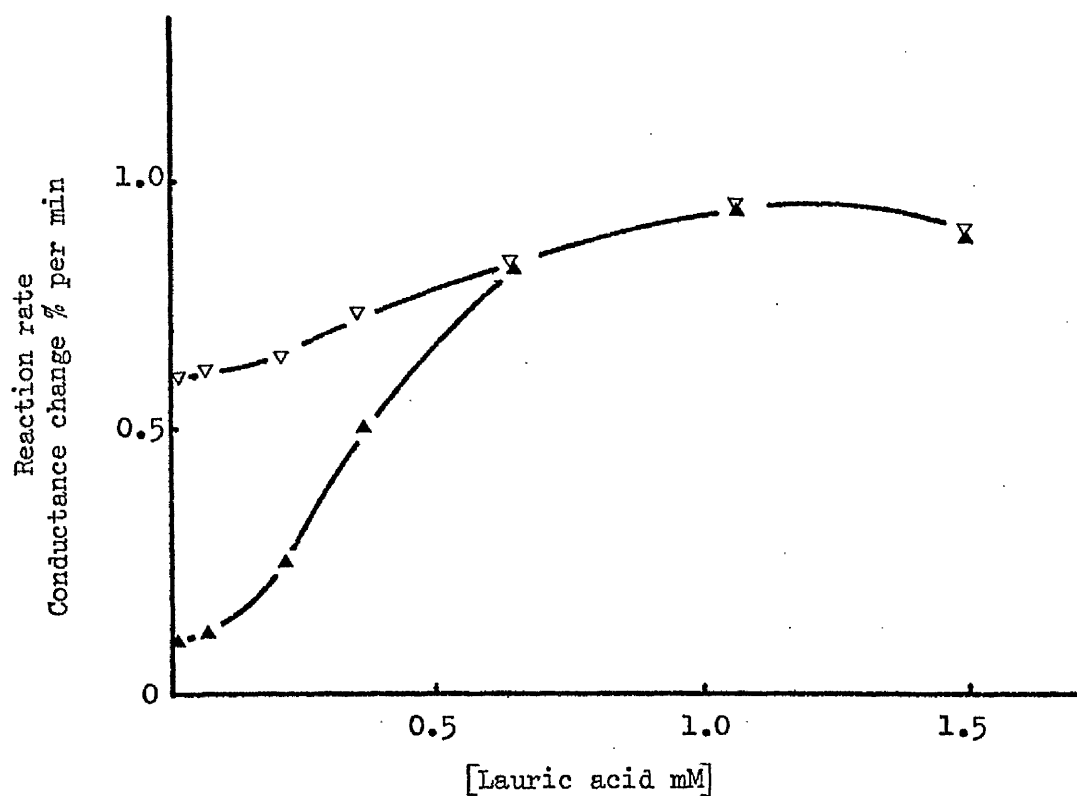


Fig (21) Activation of Bee venom PLA_2 by Lauric acid.

The initial ▲ , and max. rates ▽ of hydrolysis of dioleoyl lecithin (0.49 mM) by bee venom PLA_2 (2.5 μg per ml) plotted against lauric acid concentration, measured under the standard assay conditions at 37°C .

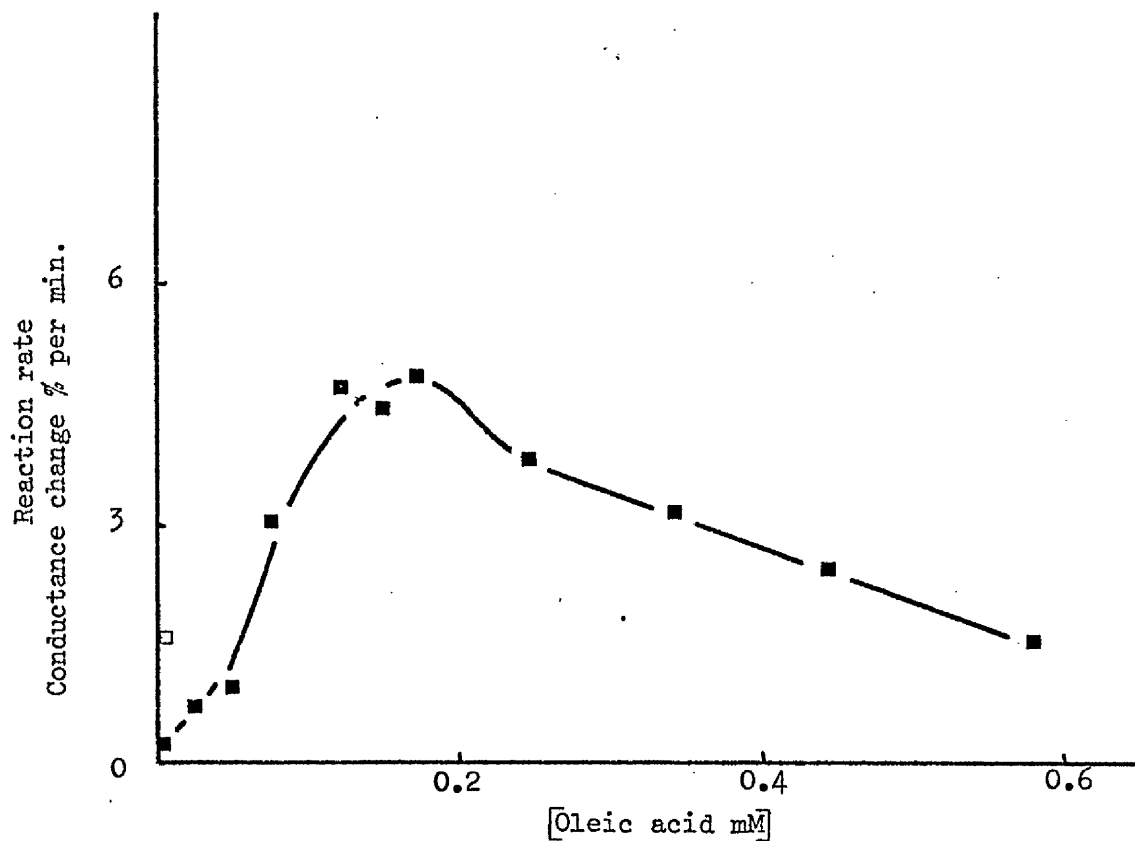


Fig (22) Activation of Bee venom PLA_2 by oleic acid.

The initial rates ■ , of hydrolysis of dioleoyl lecithin by PLA_2 (2.5 μg per ml) plotted against oleic acid concentration, measured under the standard assay conditions at 37°C □ max. rate.

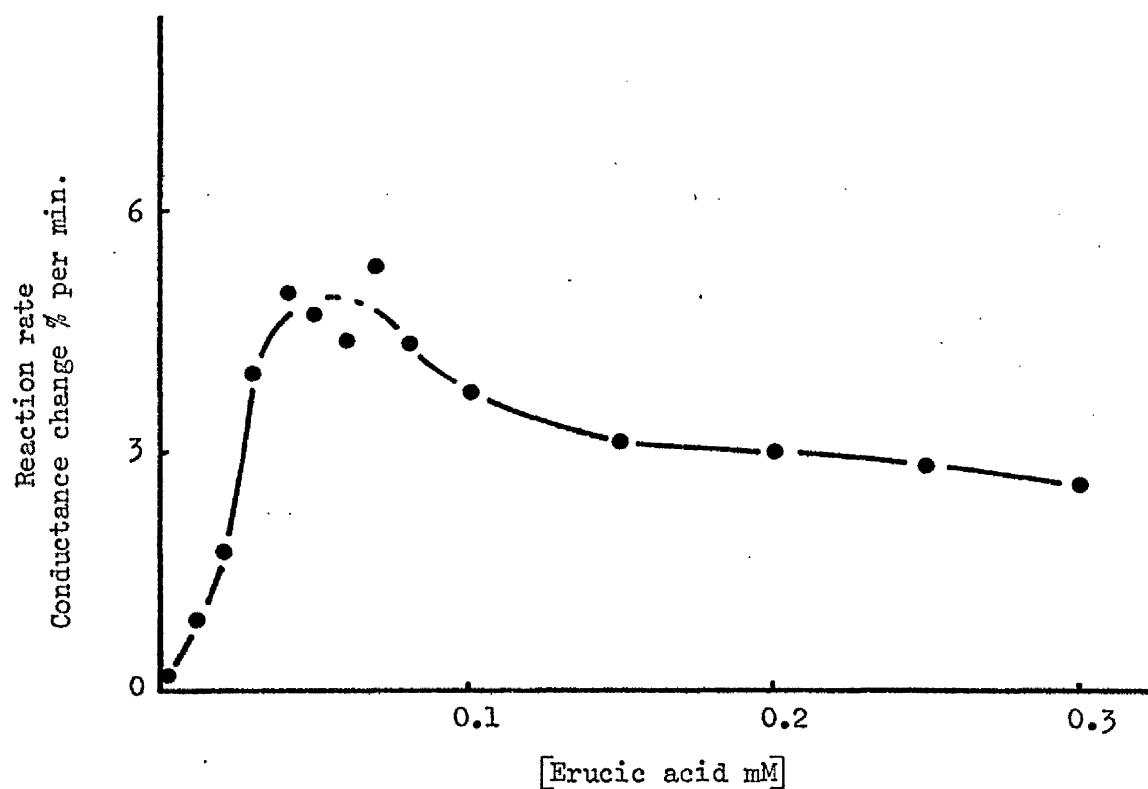


Fig (23) Activation of Bee venom PLA_2 by Erucic acid.

The initial rates of the hydrolysis of dioleoyl lecithin by PLA_2 (2.5 μg per ml) plotted against erucic acid concentration, measured under the standard assay conditions at 37°C .

TABLE 2

FATTY ACID CONC. mM	ACTIVATION FACTOR			
	0.0025	0.005	0.05	0.1
LAURIC	1.0	1.0	1.2	1.8
OLEIC	1.0	1.4	7.3	19.1
PETROSELINIC	1.0	1.3	7.4	12.9
LINOLENIC	1.0	1.12	2.6	3.6
γ -LINOLENIC	-	1.4 [‡]	4.4 [‡]	5.5 [‡]
ERUCIC	1.4	1.9	24.8	10.8
ARACHIDONIC	1.5	2.2	4.9	8.2
NERVONIC	1.5	2.0	13.25	10.6

[‡] 0.0045, 0.045, 0.09 mM

The effect of free Fatty acids on bee venom
phospholipase A₂.

Enzyme activity was measured under standard assay
conditions with fatty acid included in the assay
solution.

4.1 Fatty acid Activation

The hydrolysis curve of dioleoyl lecithin by bee venom PLA₂ in 20% propanol system (Fig 18) forms a useful assay for fatty acid activation. Monophasic curves can be produced by addition of free long chain fatty acids ($R > C_{12}$) to the reaction mixture. Free acids are apparently stronger activators than product oleic acid, but higher concentrations are required for maximum effect. Lauric acid (12:0) produces the maximum effect at 1 mM concentration in the reaction mixture (Fig 21), oleic acid (18:1) at 0.15 mM (Fig 22), erucic acid (22:1) at 0.05 mM (Fig 23). Some of the higher fatty acids like nervonic (24:1) produce the maximum effect at approximately the same concentration as erucic but are generally weaker activators. Table 2 shows the activation factors produced by free fatty acids at different concentrations. All free acids were added to 1 ml reaction solution of 20% propanol, 10 mM triethanolamine/HCl pH 8.2 containing 0.1 mM CaCl₂ and 0.24 mM dioleoyl lecithin (standard procedure). Table 2 also indicates the concentration of the free acid required for the maximum effect. Two explanations can be given for the fatty acid activation; either the fatty acids attack the substrate and increase the negative net charge (ζ -potential) of the substrate surface (Dawson, 1958), so the positive enzyme (ca 20 free-NH₃⁺ groups, Shipolini et al, 1971) can penetrate the substrate more rapidly, or the free fatty acids attack the enzyme and cause conformational changes in the protein which increases its catalytic activity. These results do not, however, allow the mechanism of action of the acids to be determined because increased chain length might facilitate interaction of the acids with either the enzyme or the substrate.

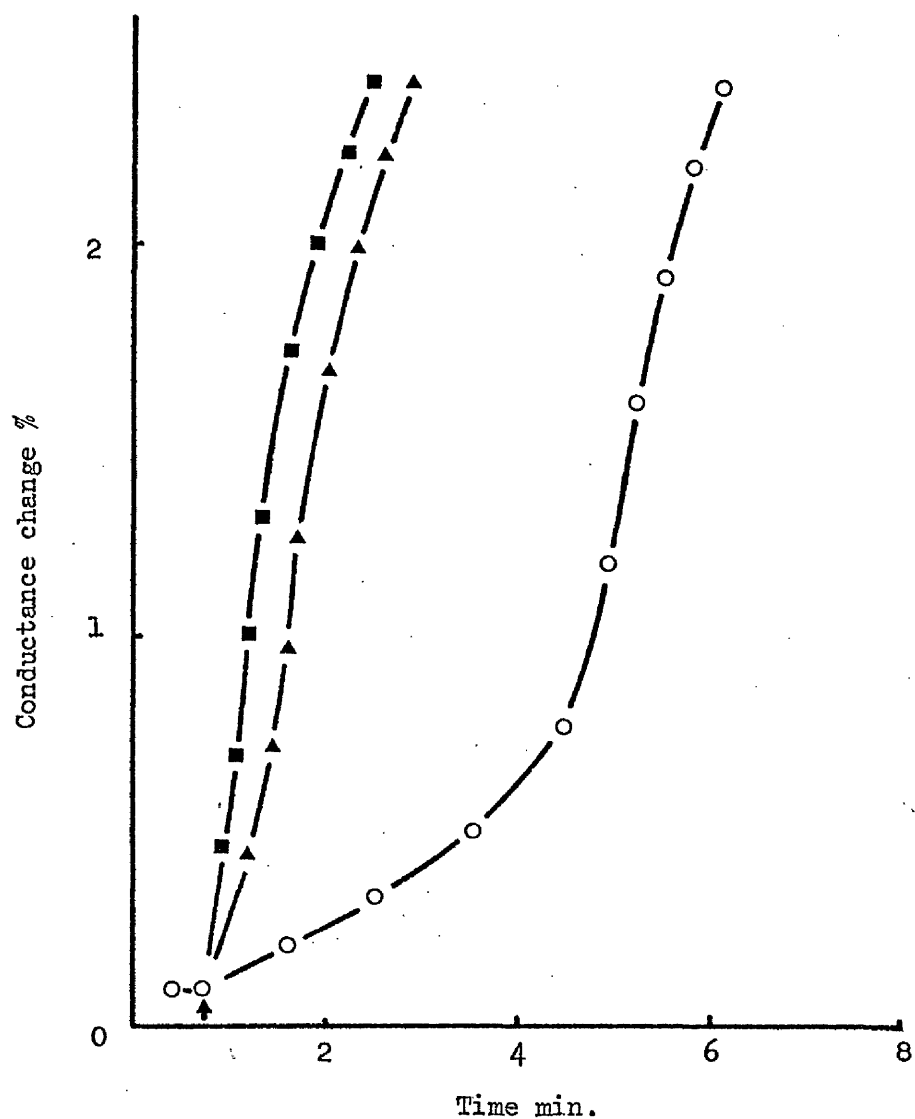


Fig (24) Activation of Bee venom PLA_2 by LEC.

The hydrolysis curves of dioleoyl lecithin (0.49 mM) by control and activated enzyme (2.5 μ g) at 37°C under the standard assay conditions.

○ control, ▲ after 2 min incubation with LEC,
 ■ after 28 min incubation with LEC.

Although free oleic acid produces the maximum effect at higher concentration than many other acids, it produces the highest levels of activation (19 fold) of all the compounds tested. This makes oleic acid the best natural activator for the bee venom PLA₂.

Because activation studies by the free fatty acids do not allow the site of action to be determined, the effect of various acylating agents on the enzyme activity was investigated. Lawrence and Moores (1975) demonstrated activation of bee venom PLA₂ by glutaraldehyde in the presence of oleic acid and by decanoic anhydride.

Further studies with fatty acid anhydrides as activators of bee venom PLA₂ gave no better results than those published by Lawrence and Moores (1975). The criteria of activation used in the present work were: a) smooth hydrolysis curve (complete activation) and b) maximum activity. Acyl ethyl carbonate esters were then tested because they are easy to prepare, of moderate reactivity and have higher solubility than the equivalent anhydrides.

4.2 The activation of Bee venom PLA₂ by Lauroyl ethyl carbonate

Preincubation of the enzyme with lauroyl ethyl carbonate (LEC) in the absence of substrate progressively eliminates the biphasic curve for hydrolysis of the long chain substrate (dioleoyl lecithin) (Fig 24). 100 µl of 1 mg/ml PLA₂ prepared in propanolic buffer were incubated at 37°C with 5 µl of 2% solution of LEC made in diethyl ether. 2.5 µl samples were withdrawn at time intervals and assayed by the standard procedure with 0.49 mM dioleoyl lecithin (Fig 24). To follow

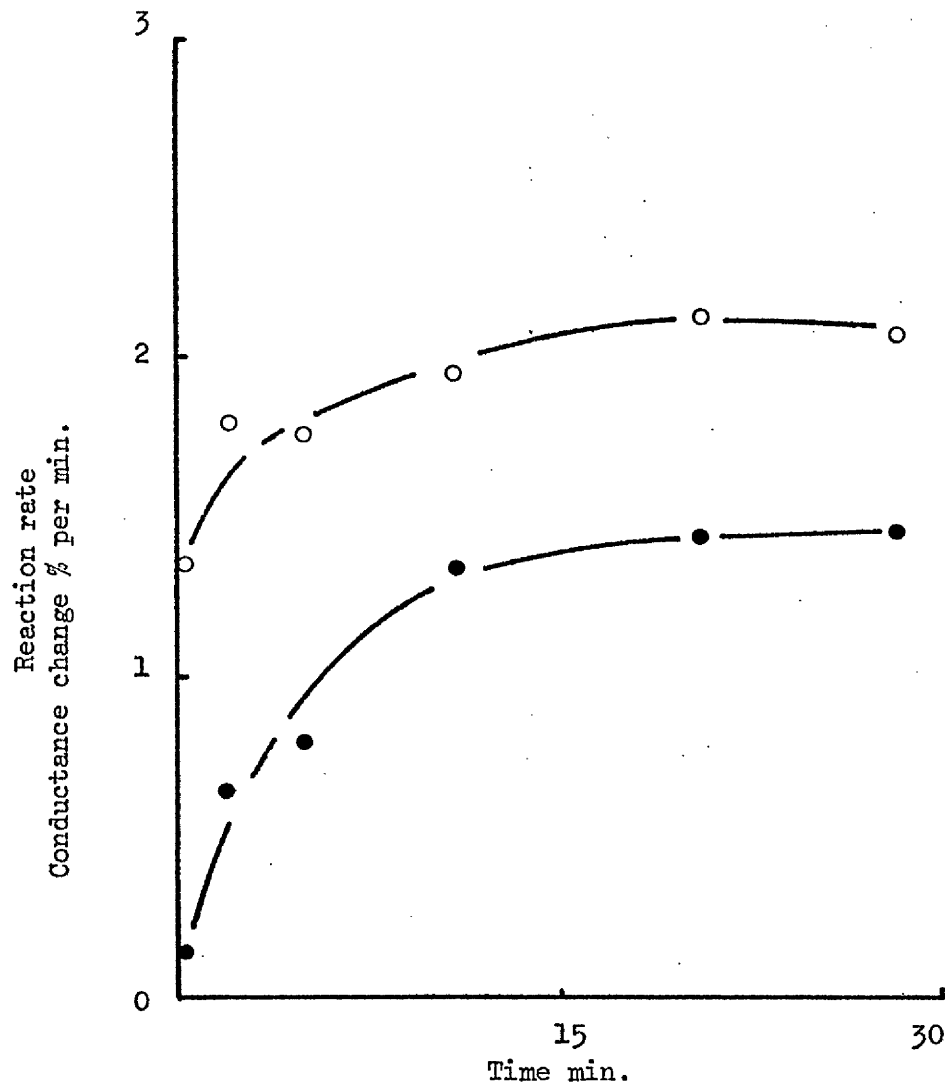


Fig (25) Activation of Bee venom PLA_2 by LEC.

Initial ● , and max. rates ○ of hydrolysis of dioleoyl lecithin (0.49 mM) by PLA_2 (2.5 μg per ml) of the fig (24), measured under the standard assay conditions at 37°C . The enzyme was activated by LEC as described in the text.

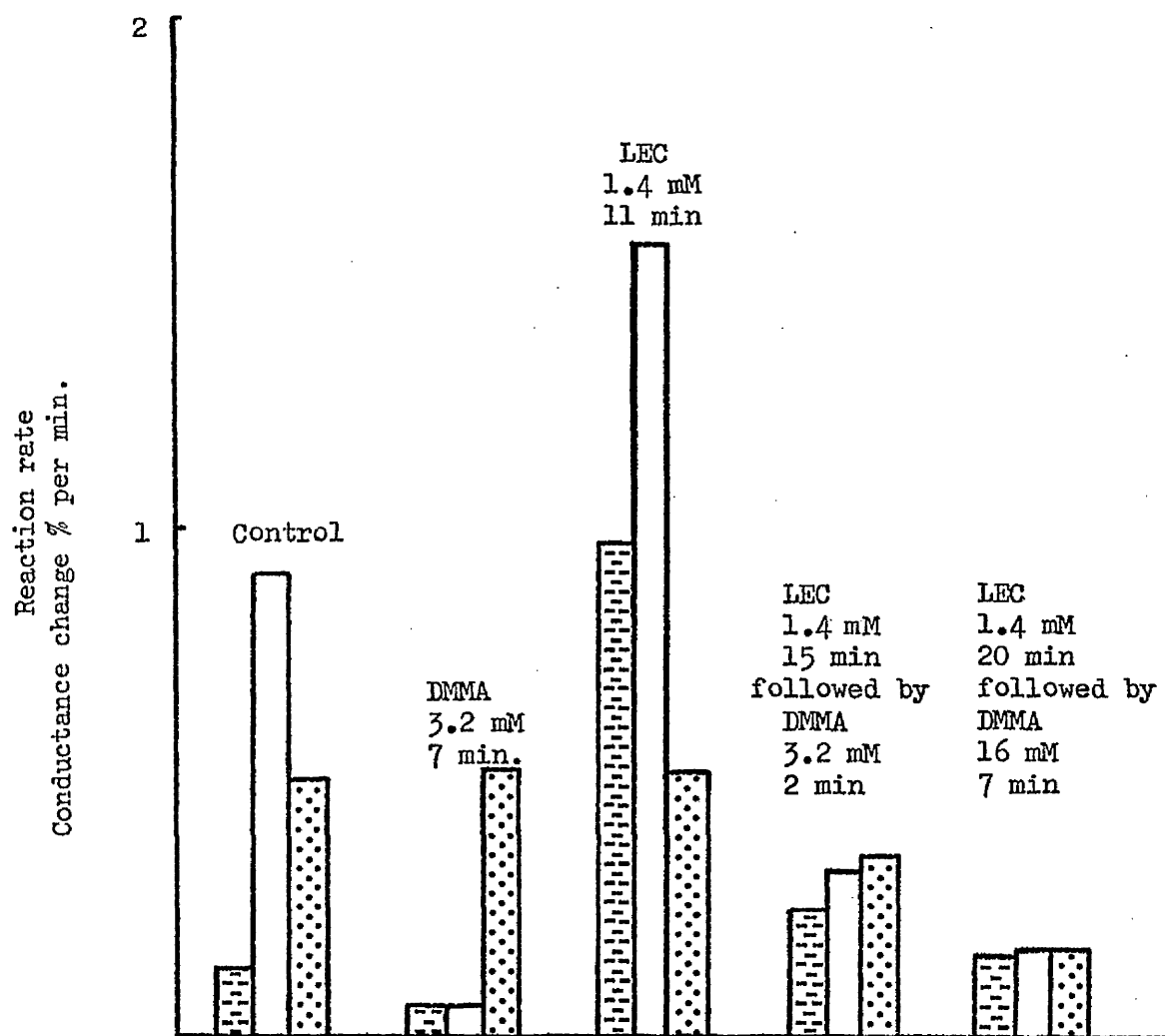





Fig (26) The effect of LEC and DMMA on bee venom PLA_2 activation.

The initial  and max. rates  of hydrolysis of dioleoyl lecithin (0.49 mM) and initial rates  of diundecenoyl lecithin (0.95 mM) by PLA_2 (2.5 μ g per ml), measured under the standard condition at 37°C.

The enzyme (1 mg/ml) was preincubated with LEC and/or DMMA as described in the text.

the activation of the enzyme by LEC during the time of its incubation with the reagent the initial and late rates of the hydrolysis curves of the Fig (24) were plotted against the incubation time (Fig 25). The maximum activation of the enzyme occurs within 20 min (Fig 25). This activation by LEC has little effect with short-chain substrate (diundecenoyl lecithin) (Fig 26).

Preincubation of the enzyme with dimethyl maleic anhydride (DMMA) also gives a smooth hydrolysis curve for long chain substrate but acts by inhibiting the late phase of the hydrolysis of dioleoyl lecithin rather than activating the early phase (Fig 26). 100 μ l of 1 mg/ml PLA_2 prepared in 100 mM triethanolamine/MOPS, pH 8.2, containing 20% n-propanol and 0.1 mM CaCl_2 were incubated at 37°C with 2.5 μ l of 1% solution of DMMA made in diethyl ether. DMMA reacts with the enzyme rapidly and inhibits the late phase of the reaction (Fig 26). The enzyme treated with DMMA recovers its activity very rapidly at low pH (Butler et al, 1967). High concentration of the reagent lowered the pH to a level where the enzyme recovered activity very quickly; to overcome this, buffer concentration was increased to 100 mM.

Enzyme activated with LEC and then treated with DMMA retains the near linear curve for the hydrolysis of dioleoyl lecithin, but the activity is reduced at least 5-fold (Fig 26). This treatment also reduces the activity towards diundecenoyl lecithin and at higher concentrations of DMMA hydrolysis of both substrates is monophasic at the same reaction rate.

Activation by LEC cannot be ascribed to free lauric acid,

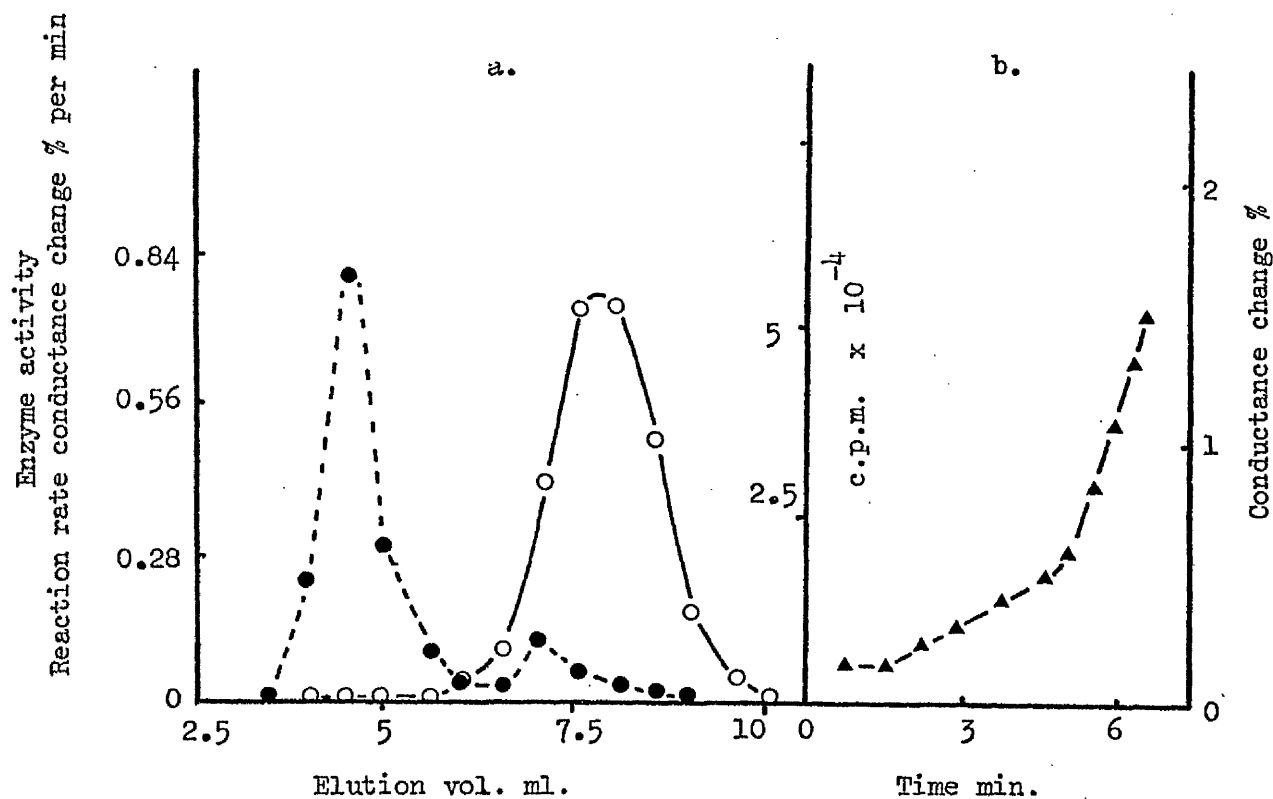


Fig (27) Bio-Gel P-6 column chromatography of control Bee venom PLA₂, mixed with [¹⁴C]-lauric acid.

a) Enzyme activity determined using diundecenoyl lecithin as substrate (0.95 mM) ● , and radioactivity O .

b) The hydrolysis of dioleoyl lecithin (0.49 mM) by the most active enzyme fraction in the 20% propanol assay system.

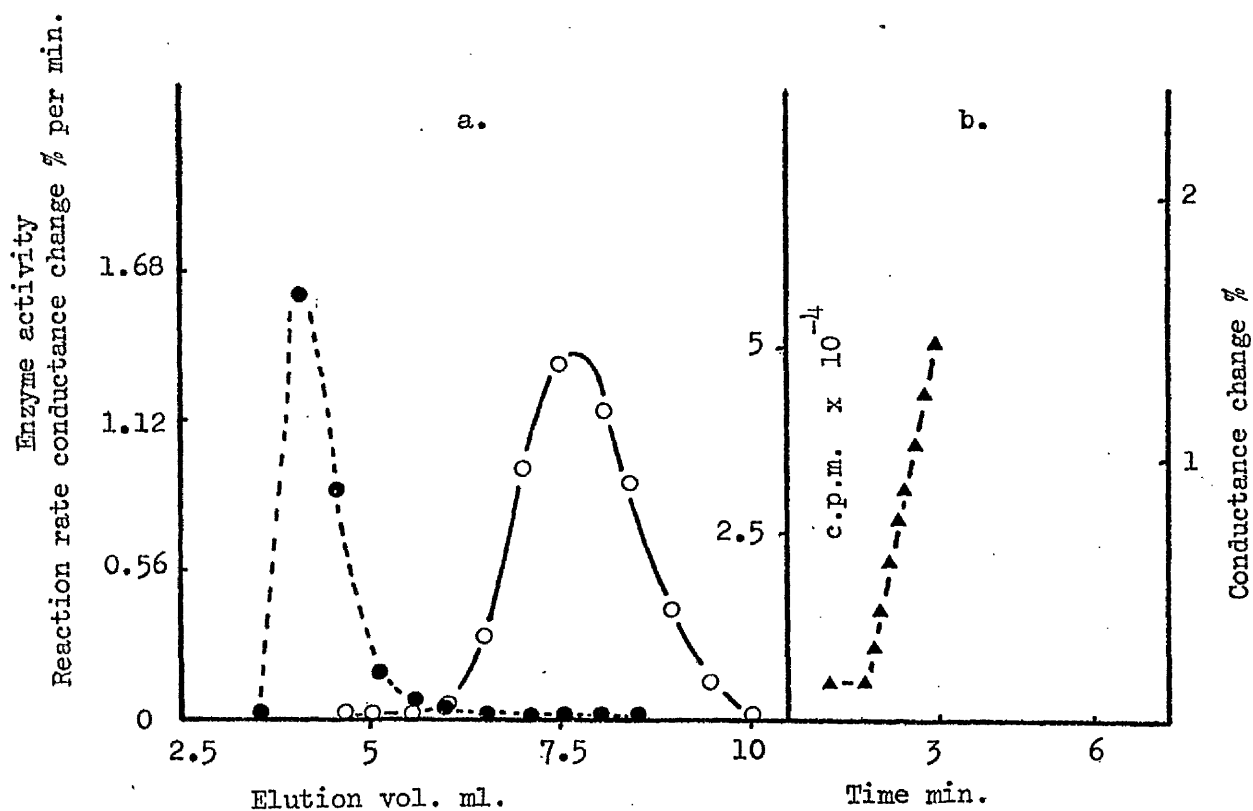


Fig (28) Bio-Gel P-6 column chromatography of LEC preactivated PLA₂, mixed with [¹⁴C]-lauric acid

- a) Enzyme activity determined using diundecenoyl lecithin as substrate (0.95 mM) ● , and radioactivity ○ .
- b) The hydrolysis of dioleoyl lecithin (0.49 mM) by the most active fraction in the 20% propanol assay system.

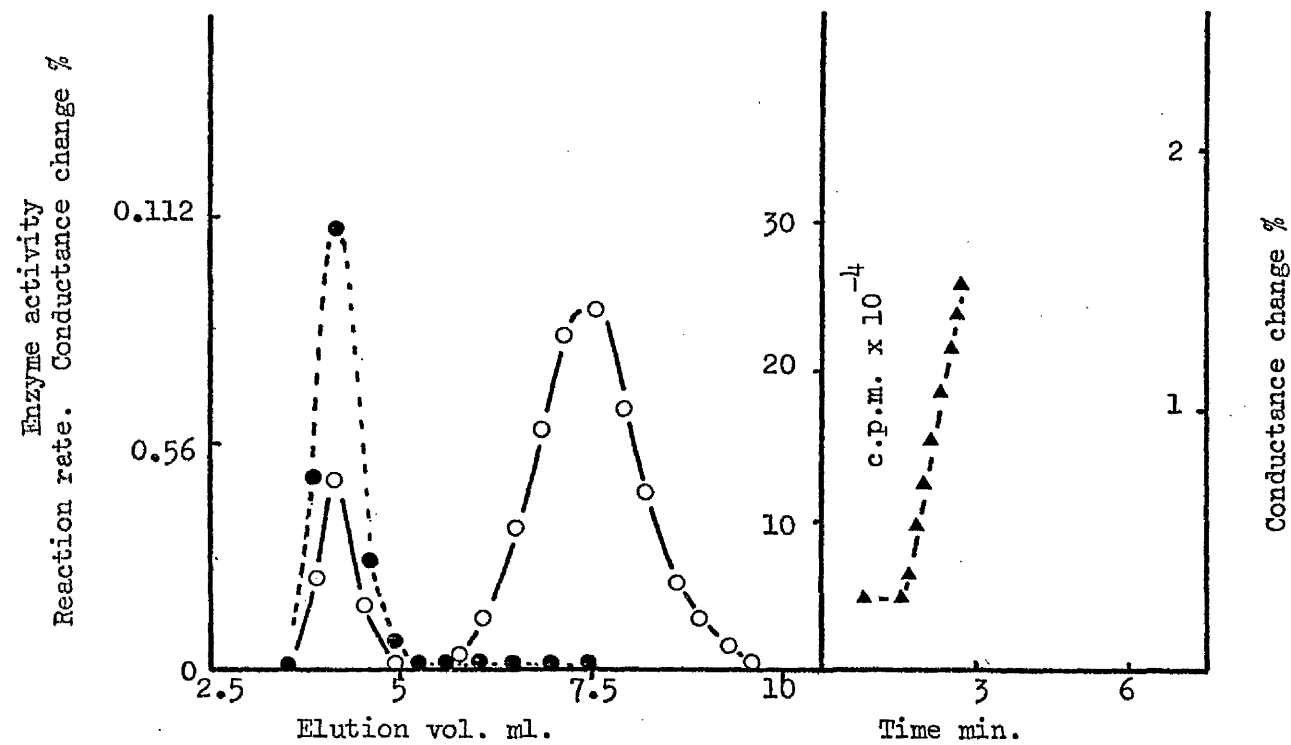


Fig (29) Bio-Gel P-6 column chromatography of $[^{14}\text{C}]$ -LEC preactivated PLA_2 .

- a) Enzyme activity determined using diundecenoyl lecithin as substrate (0.95 mM) ● , and radioactivity ○ .
- b) The hydrolysis of dioleoyl lecithin (0.49 mM) by the most active fraction. in the 20% propanol assay system.

because controls in which LEC was replaced by the equivalent concentration of lauric acid showed no activation. But definite proof was obtained using gel filtration to separate unactivated enzyme and enzyme activated by both "cold" and $[^{14}\text{C}]$ -labelled LEC from tracer lauric acid or LEC. 250 μl of 1 mg/ml bee venom PLA_2 prepared in propanolic buffer (ca 0.017 μMoles protein) were incubated at 37°C with 0.18 μMol $[^{14}\text{C}]$ -lauric acid (specific activity 14 $\mu\text{Ci}/\mu\text{Mol}$) and passed through a column (12 x 105 mm) of BIO-Gel P-6 equilibrated in the propanolic buffer (Fig 27). The same amount of enzyme was treated with 0.18 μMoles LEC for 12 min and 0.18 μMoles of $[^{14}\text{C}]$ -lauric acid were added and the mixture was passed through the same column (Fig 28). Again the same amount of enzyme was treated with 0.18 μMol of $[^{14}\text{C}]$ -LEC (see Materials and Methods) and passed through the same column (Fig 29). Figs 27 and 28 show that neither the unactivated nor the activated by LEC enzyme bind free lauric acid, the enzyme activity is well separated from the radioactivity. Fig 29 shows that enzyme activated by ^{14}C -labelled LEC now has bound radioactivity. Samples from all the column fractions were assayed at 37°C in 1 ml 10 mM triethanolamine/ Cl^- , pH 8.2, containing 20% n-propanol and 0.1 mM CaCl_2 with dihexanoyl lecithin as substrate.

The activated enzyme retains full activation after the treatment and is stable in this form indefinitely (Fig 29). Enzyme activated with $[^{14}\text{C}]$ -labelled LEC became labelled corresponding to uptake of 1.8 lauroyl residue per enzyme molecule.

These experiments show definitely that the acylating agent

attacks the enzyme and is highly specific for the activating site, and not by releasing fatty acids which they interact with the substrate. The activated enzyme is free from traces of unreacted lauroyl ethyl carbonate and fatty acid after the column treatment, but it retains its full activation which means that the fatty acid residue is bound to the protein by a stable bond.

The degree of activation produced by lauroyl ethyl carbonate is higher than that produced by anhydrides. Activation by free acids increases with increasing side-chain length and the lauroyl residue was chosen for this study only to minimise direct activation by free acid.

The action of dimethyl maleic anhydride on untreated enzyme shows that activation can be blocked without generally inactivating the enzyme, but in contrast it gives general inactivation for covalently activated enzyme. These results suggest that the dimethyl maleic anhydride attacks both active and activating site of the protein.

4.3 The effect of different acylating agents on the activation of Bee Venom PLA₂

LEC proved to be a good activating agent, but it failed to eliminate the biphasic character of the hydrolysis curve of dioleoyl lecithin by the bee venom PLA₂ in the 20% n-propanol system. The low level of acylation (1.8 molecules per enzyme molecule) suggest that only one residue binds to the activating site, although these results do not eliminate the possibility that two residues are required.

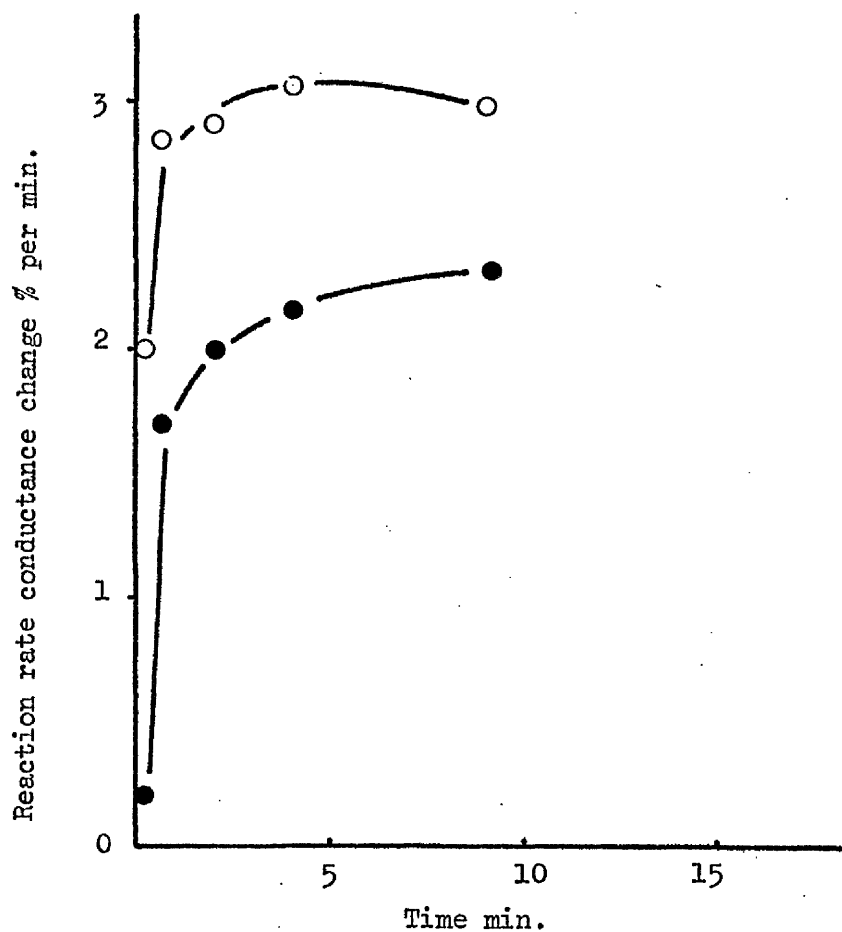


Fig (30) Activation of Bee venom PLA_2 by lauroyl chloride.

Enzyme (1 mg per ml) was incubated with the lauroyl derivative (1 mM) at 37°C and $2.5\ \mu\text{l}$ samples withdrawn for assay at measured time intervals.

Initial ● and maximum O rates of the hydrolysis of dioleoyl lecithin (0.24 mM) by bee venom PLA_2 ($2.5\ \mu\text{g}$ per ml) measured under standard assay conditions at 37°C .

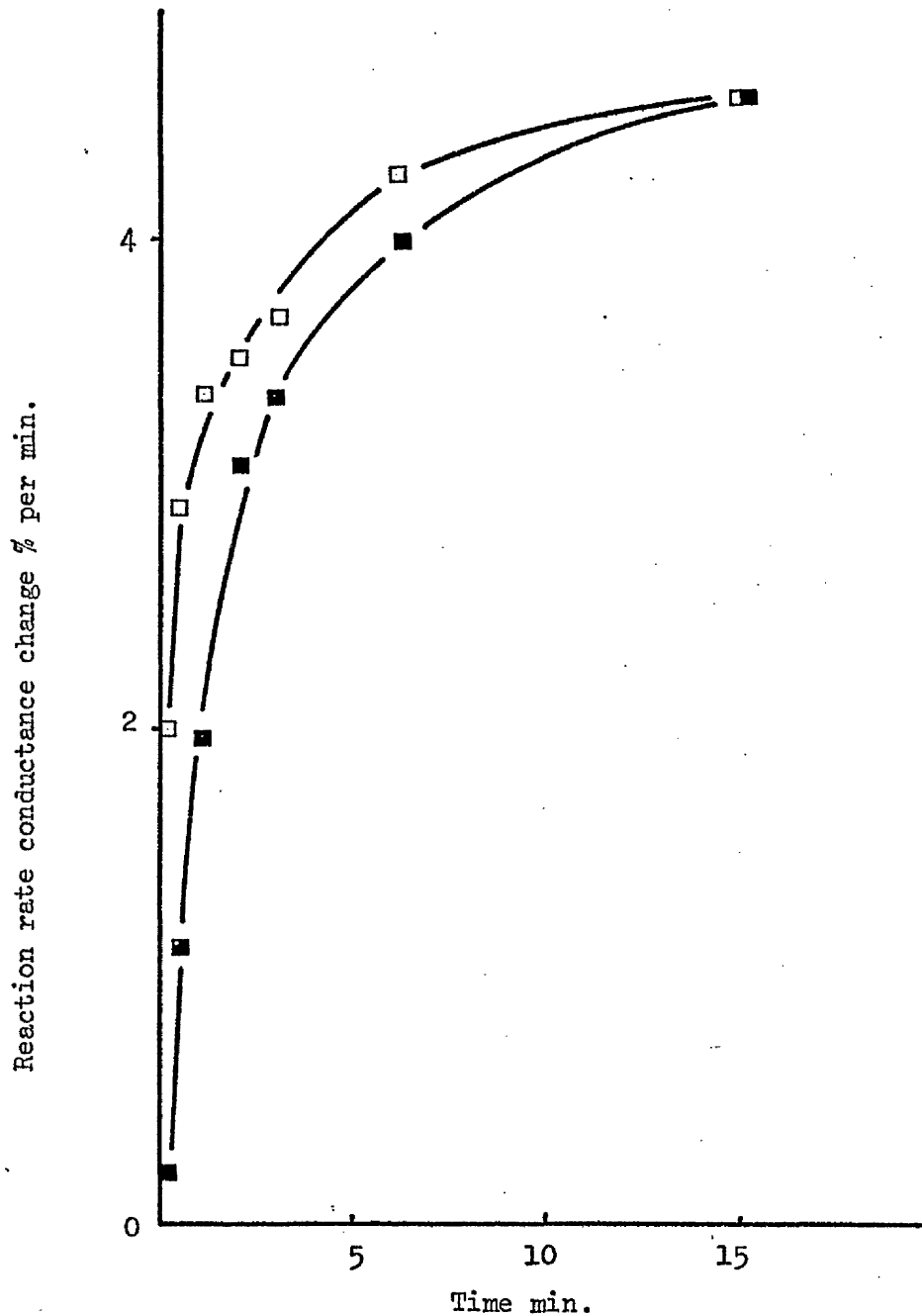


Fig (31) Activation of Bee venom PLA_2 by lauroyl azide.

Enzyme (1 mg per ml) was incubated with the lauroyl derivative (1 mM) at 37°C and 2.5 μl samples withdrawn for assay at measured time intervals.

Initial ■ and maximum □ rates of the hydrolysis of dioleoyl lecithin (0.24 mM) by bee venom PLA_2 (2.5 μg per ml) measured under standard assay conditions at 37°C .

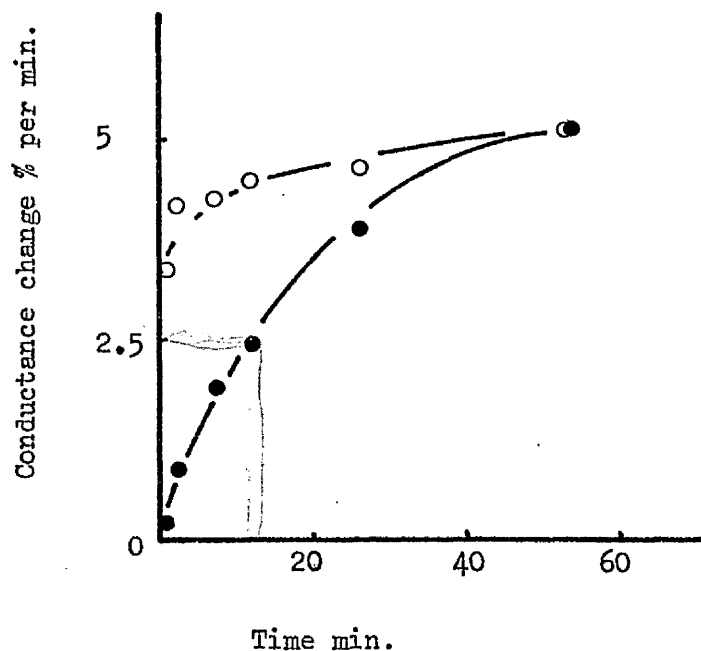


Fig (32) Activation of Bee venom PLA_2 by lauroyl imidazolid.

Enzyme (1 mg per ml) was incubated with the lauroyl derivative (1 mM) at 37°C and 2.5 μl samples withdrawn for assay at measured time intervals.

Initial ● and maximum O rates of the hydrolysis of dioleoyl lecithin (0.24 mM) by bee venom PLA_2 (2.5 μg per ml) measured under standard assay conditions at 37°C .

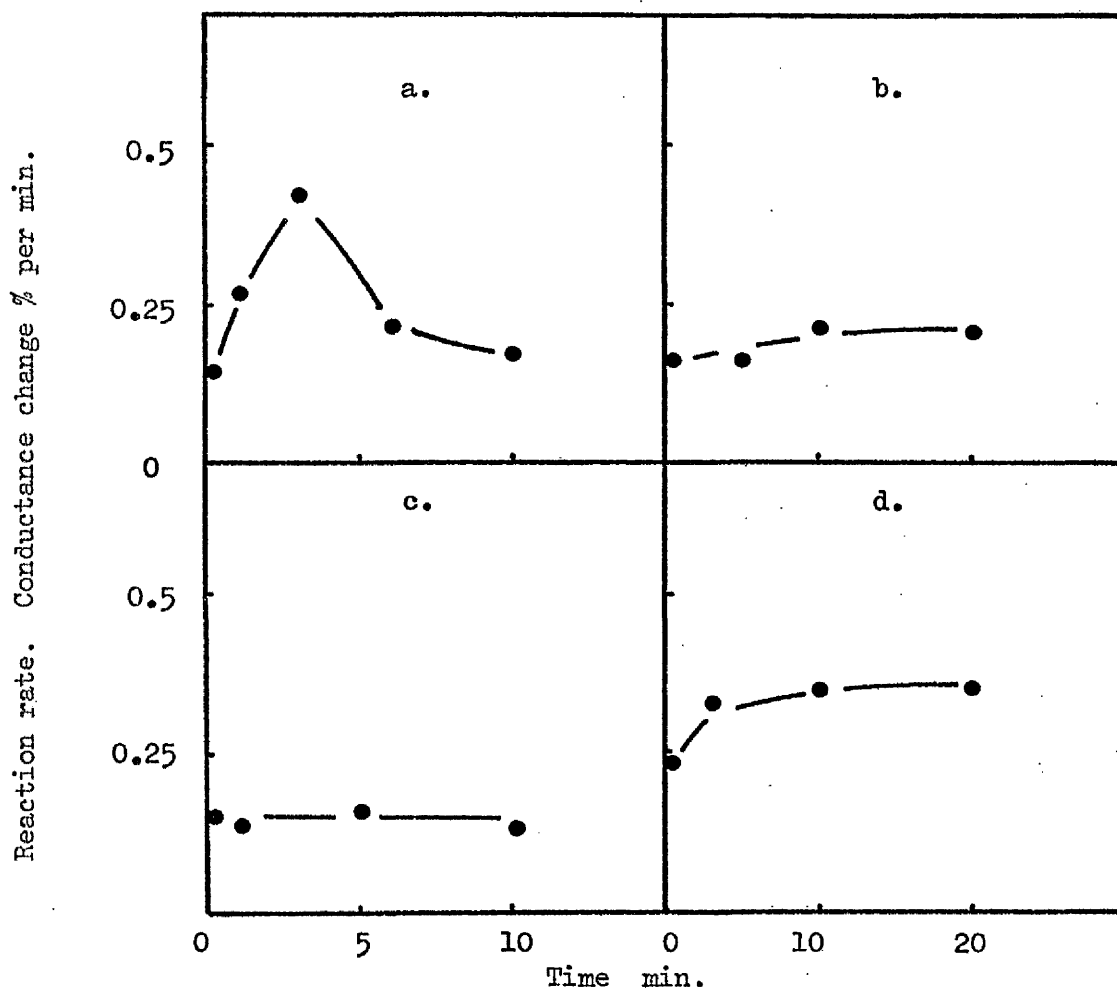


Fig (33) Activation of Bee venom PLA₂ by lauroyl derivatives.

The enzyme (1 mg per ml) was incubated with 1 mM of a) Lauroyl thio-glycolate, b) lauroyl acetonitrile, c) lauroyl sulfate, d) lauroyl 1 cyclohexyl-3(Morpholino-ethyl)-carbodiimide at 37°C and 2.5 µl samples withdrawn for assay at measured time intervals.

Initial rates of hydrolysis of deoleoyl lecithin (0.24 mM) by bee venom PLA₂ (2.5 µg per ml) measured under standard assay conditions at 37°C.

TABLE 3

LAUROYL-DERIVATIVE (1 mM)	ACTIVATION FACTOR	$\frac{1}{2}$ MAXIMUM ACTIVATION Min.
LAUROYL-AZIDE	20.6	1.2 ± 0.5
" -CHLORIDE	10.0	0.2 ± 0.6
" -ETHOXYFORMATE	11.8	4.0 ± 0.5
" -IMIDAZOLIDE	25.0	12.0 ± 0.5
" -THIO-GLYCOLATE	1.4	-
" -CARBODIIMIDE	1.4	-
" -ACETONITRILE	1.2	-
" -SULPHATE	1.0	-

The effect of Lauroyl derivatives on bee venom phospholipase A₂.

The enzyme (1 mg/ml) was incubated with lauroyl derivatives (1 mM) in assay buffer at 37°C.

Samples (2.5 μ l) were withdrawn and assayed by the standard procedure and initial rates were measured at 37°C.

Therefore a variety of other derivatives of lauric acid were synthesized to find more selective activators.

The enzyme (1 mg/ml) prepared in propanolic buffer, was treated with these acylating agents (1.0 mM), and assayed by the standard procedure.

Lauroyl chloride did not produce full activation, as judged from the linearity of the early phase of substrate hydrolysis curves (Fig 30), and treatment with excess reagent was inhibitory. Lauroyl azide gives complete activation of the enzyme judged by higher activity levels than product activation alone (Fig 31). Lauroyl imidazolidine also gives complete activation of the enzyme and produces even higher activity levels than the azide (Fig 32). Lauroyl thio-glycolate, lauroyl acetonitrile, lauroyl 1 cyclohexyl-3(2-morpholinoethyl)carbodiimide and lauroyl sulphate produced little or no activation (Fig 33).

Table 3 summarizes all the results obtained with the different acylating agents giving the activation factor and the time in which the half maximum activation of the enzyme occurs. Lauroyl chloride, lauroyl azide and lauroyl imidazolidine proved to be the most effective compounds tested (Table 3). Lauroyl chloride reacts rapidly with the enzyme, but is readily hydrolyzed by water and it is not as good as LEC. Lauroyl azide reacts with the enzyme rather slower than the chloride, but the hydrolysis of this reagent in water is slower, however it is very unstable even at temperatures below 0°C and it decomposes in a few hours. Lauroyl imidazolidine reacts with the enzyme more slowly than the azide, but it produces the highest levels of enzyme activation;

TABLE 4

FATTY ACID IMIDAZOLIDES	ACTIVATION FACTOR	$\frac{1}{2}$ MAXIMUM ACTIVATION Min.
ACETYL-	1.0	-
BUTYRYL-	1.0	-
HEXANOYL-	1.2	-
OCTANOYL-	1.2	-
CAPRYL-	> 7.6	> 25
LAUROYL-	25.0	12.0 \pm 0.5
MYRISTOYL	> 16.6	> 20
STEAROYL-	> 9.5	> 45
OLEOYL-	53.3	3.0 \pm 0.5
PETROSELINOYL-	24.0	2.0 \pm 0.5
ELAIDOYL-	31.6	4.5 \pm 0.5
LINOLEOYL-	47.6	6.0 \pm 0.5
LINOLENOYL-	21.0	2.0 \pm 0.5
γ -LINOLENOYL-	26.0	2.0 \pm 0.5
ERUCOYL-	29.0	2.0 \pm 0.5
ARACHIDONOYL	19.7	1.5 \pm 0.5
NERVONOYL	27.9	2.5 \pm 0.5

The effect of Fatty acid imidazolides on bee venom phospholipase A₂.

The enzyme (1 mg/ml) was incubated with fatty acid imidazolides (1 mM) in assay buffer at 37°C. Samples (0.5 μ l) were withdrawn and assayed by the standard procedure and initial rates were measured at 37°C.

it is stable in water and there was no detectable hydrolysis of the reagent within a few hours.

These results and the fact that "imidazolidine" was a very easy compound to synthesize (see Materials and Methods) led us to study its action on the enzyme in more detail.

4.4 The activation of Bee venom PLA₂ by acyl imidazolides

4.4.1 The effect of acyl chain length

A range of acyl imidazolides was prepared to test the effect of side chain length and unsaturation on enzyme activation. Table 4 shows that long-chain imidazolides ($R > C_{10}$) are more effective, and also react considerably faster than the short-chain imidazolides ($R \leq C_{10}$). Among the long-chain imidazolides oleoyl and linoleoyl are the most effective, although not the fastest acting. Oleoyl imidazolidine gives the highest activation factor (53 fold) ever found for the activation of bee venom PLA₂ in this assay.

In all cases the enzyme (1 mg/ml) was activated by acyl imidazolides (1 mM) and assayed by the standard procedure.

Pure lauroyl and oleoyl imidazolidine were tested for hydrolysis and aminolysis in the conductimetric assay with acetyl imidazolidine used as a control. In no case could any reaction be seen although the acetyl derivative behaved as expected.

To test whether the imidazole group conferred special

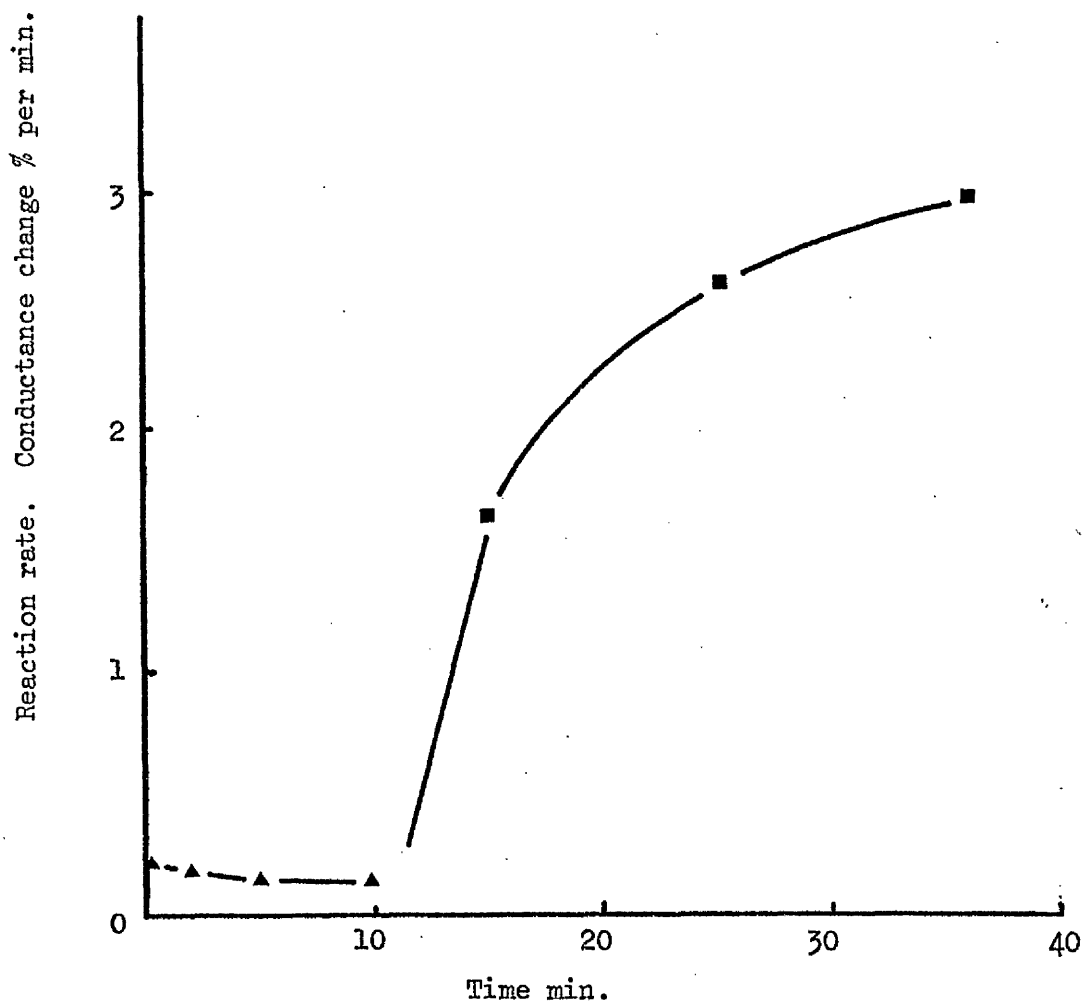


Fig (34) Activation of Bee venom PLA_2 by acetyl imidazolide followed by oleoyl imidazolide.

The enzyme (1 mg/ml) was incubated with acetyl imidazolide (4.5 mM) ▲, followed by oleoyl imidazolide (1 mM) ■ (after 11 min) at 37°C and 2.5 μl samples withdrawn for assay at measured time intervals. The initial rates of hydrolysis of dioleoyl lecithin (0.24 mM) by bee venom PLA_2 (2.5 μg per ml) measured under standard assay conditions at 37°C .

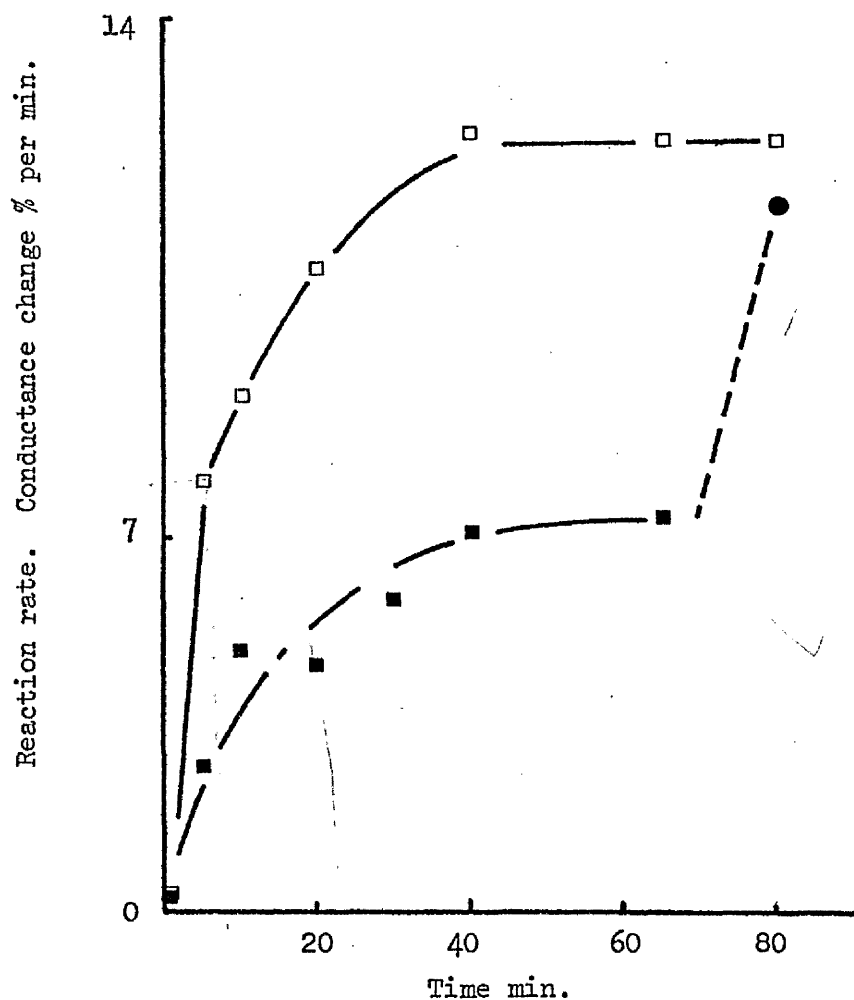


Fig (35) Parallel activation of Bee venom PLA_2 by lauroyl imidazolide and oleoyl imidazolide.

The enzyme (1 mg/ml) was incubated with oleoyl \square or lauroyl \blacksquare imidazolides (1 mM) at 37°C .

After 70 min oleoyl imidazolide (1 mM) \bullet was added to lauroyl imidazolide enzyme.

The initial rates of hydrolysis of dioleoyl lecithin (0.24 mM) by bee venom PLA_2 (2.5 μg per ml) measured under standard assay conditions at 37°C .

reactivity towards the activation centre of the enzyme, treatment with acetyl imidazolide was followed by incubation with oleoyl imidazolide. The acetyl derivative (4.5 mM) produced partial inactivation (30% in 10 min) (Fig 34), but the residual activity showed the typical biphasic assay curve. Addition of 1 mM oleoyl imidazolide to the acetyl imidazolide-enzyme mixture activated this residual activity by a factor of 21-fold (Fig 34). Also incubation of the enzyme with carbonyldiimidazole gave no activation or inhibition. Competitive experiments using lauroyl and oleoyl imidazolides confirmed that the oleoyl derivative reacted more rapidly in addition to producing higher activity levels. Enzyme was incubated with lauroyl imidazolide and after 70 min incubation oleoyl imidazolide was added to the mixture (Fig 35) or the enzyme was incubated with a mixture of lauroyl and oleoyl imidazolides. In both experiments the level of enzyme activation was as high as that produced by oleoyl imidazolide alone. These results suggest that the bound lauroyl residue gives very similar activation to the bound oleoyl residue and therefore the lauroyl derivative appears to activate less because it reacts much more slowly.

The selectivity of these reagents therefore appears to be determined by the acyl side chain and the low reactivity of the head group ensures that little non-specific acylation occurs.

Long chain saturated fatty acid derivatives show anomalous behaviour, but they appear to be insoluble in the incubation medium (Table 4).

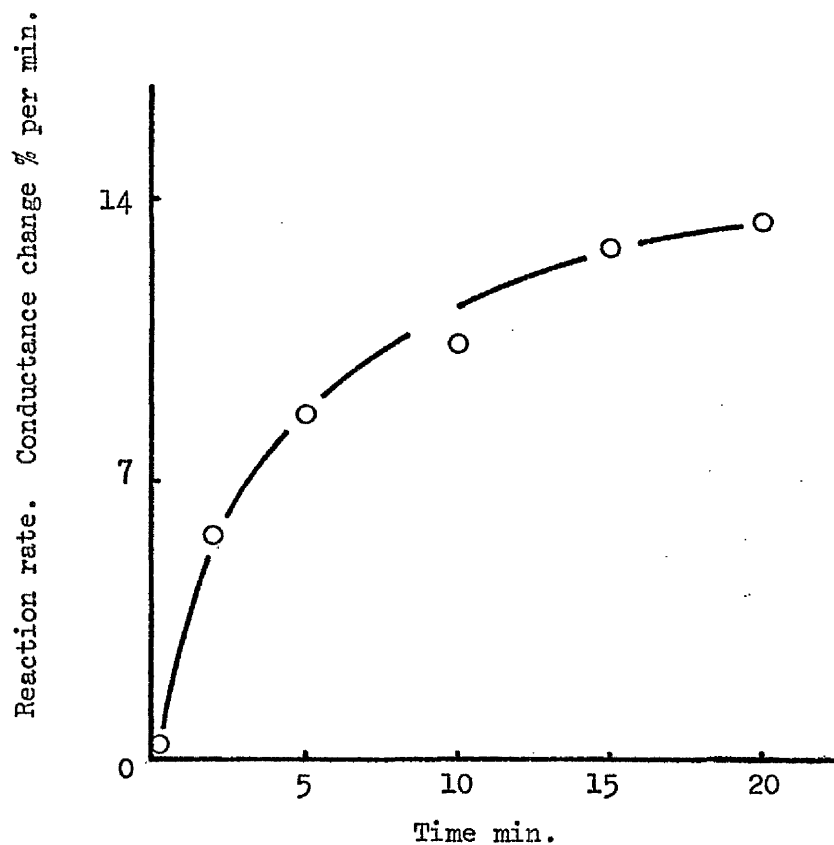


Fig (36) Activation of Bee venom PLA_2 by oleoyl imidazolide.

Enzyme (1 mg/ml) was incubated with oleoyl imidazolide (1 mM) at 37°C and 0.5 μl samples withdrawn for assay at measured time intervals. Initial rates of hydrolysis of dioleoyl lecithin (0.24 mM) by bee venom PLA_2 (2.5 $\mu\text{g}/\text{ml}$) measured under standard assay conditions at 37°C .

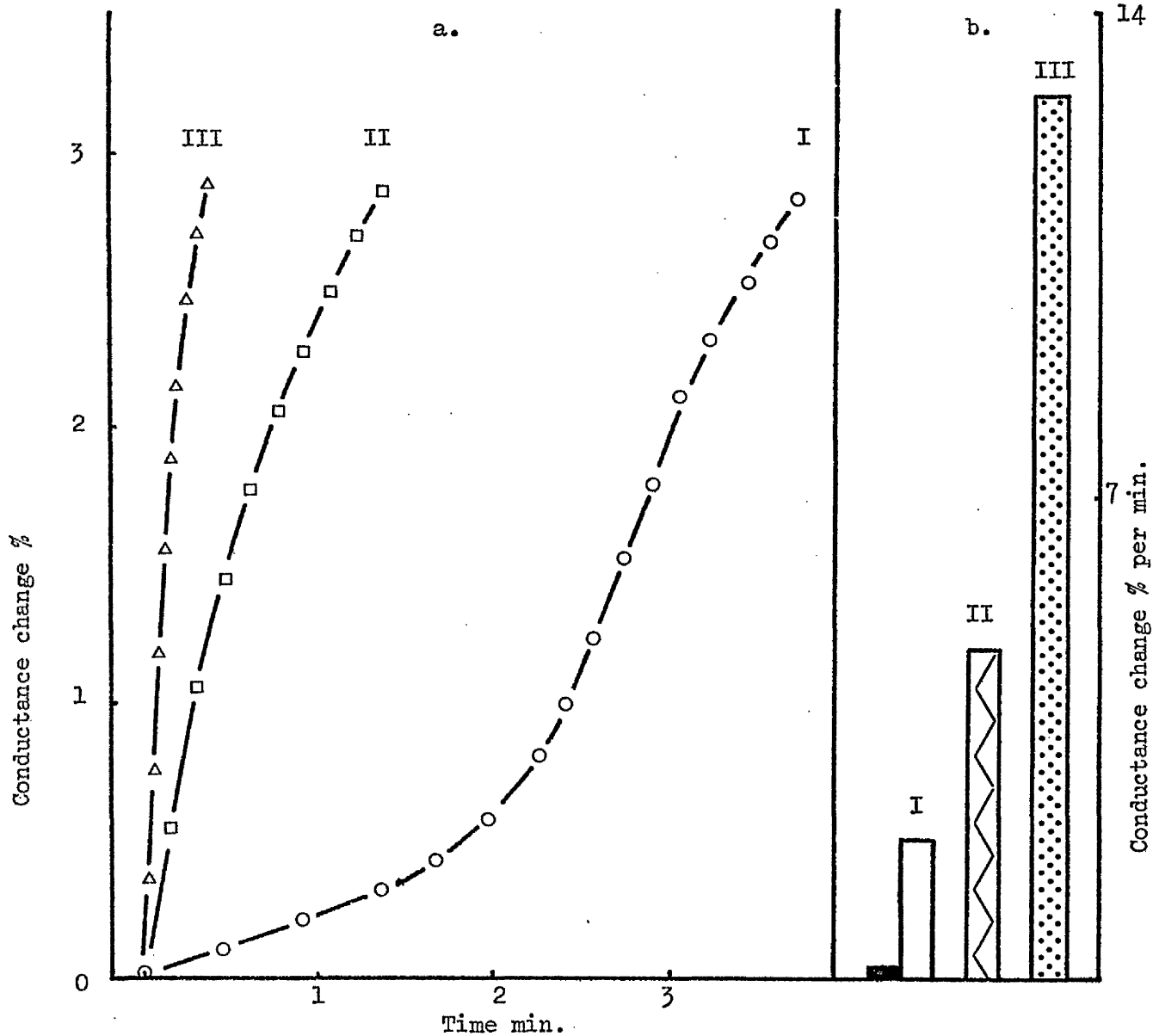


Fig (37) Comparison of Oleoyl imidazolid and free oleic acid activated enzyme.

a) The hydrolysis of dioleoyl lecithin (0.24 mM) by bee venom PLA₂ (2 µg per ml) at 37°C measured under standard assay conditions.

I, untreated enzyme; II assayed with 0.17 mM oleic acid; III the enzyme (1 mg/ml) was pre-activated with 1 mM oleoyl imidazolid, incubated for 20 min and diluted 2,500 fold for assay.

b) Initial and maximum rates obtained from Fig 37a ,
 ■, ◻ and ◻ initial rates from I, II and III respectively.

◻ maximum rates from I.

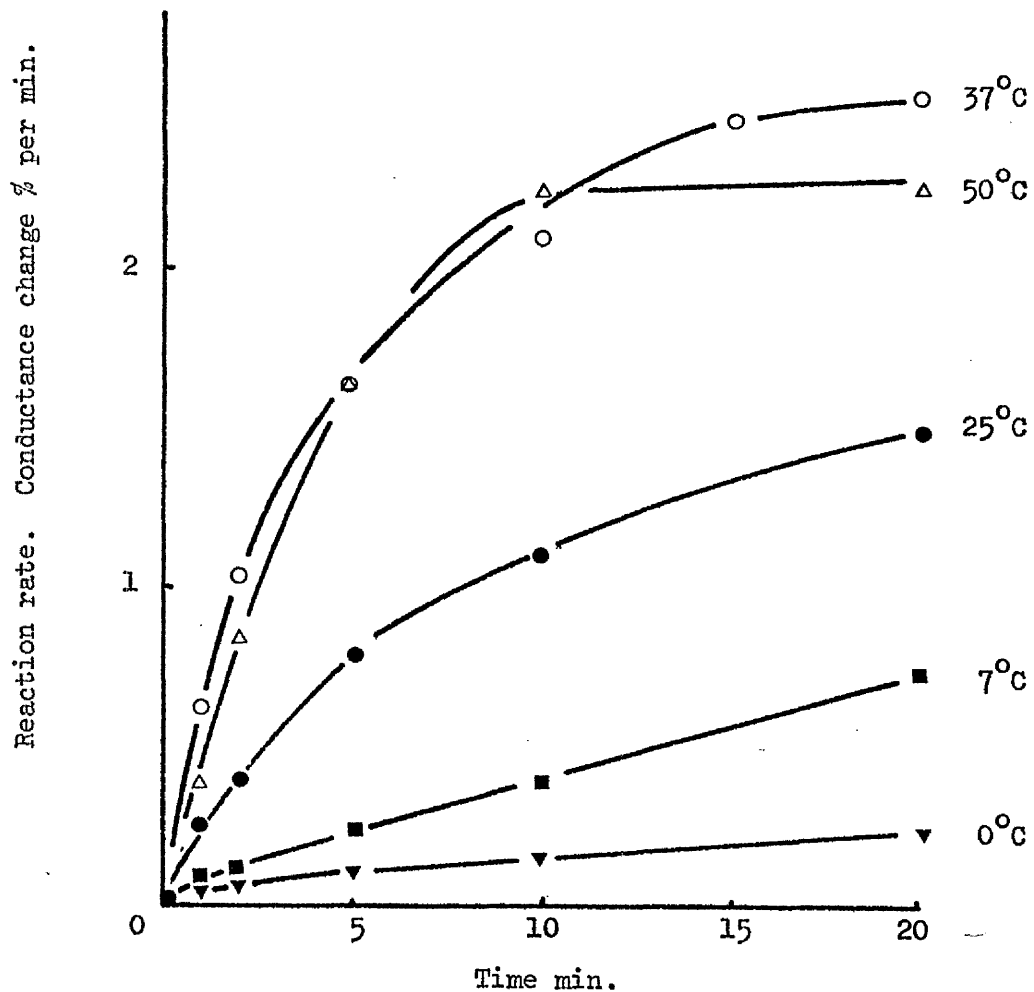


Fig (38) The effect of temperature on the rate of activation of Bee venom PLA₂ by oleoyl imidazolide.

The enzyme (1 mg/ml) was incubated with oleoyl imidazolide (0.6 mM) diluted 2,000 fold for assay and initial rates of the hydrolysis of dioleoyl lecithin (0.24 mM) by bee venom PLA₂ (0.5 µg per ml) at 37°C measured under standard assay conditions.

Oleoyl imidazolidate activates the enzyme when incubated in 1:1 molar proportion with the enzyme although rates are rather low.

Because oleoyl imidazolidate produced the best results it was used for all further work and routinely produced maximal activation factors of 50-fold with 8-10 fold stimulation of maximum product activation levels (Fig 36). Fig 37 shows the comparison of the activation of the enzyme by free oleic acid and oleoyl imidazolidate.

4.4.2 Temperature dependence of activation

To test whether the temperature affects the speed of enzyme activation by oleoyl imidazolidate, the enzyme was activated within the temperature range of 0°C to 50°C and assayed at 37°C.

The enzyme (1 mg/ml) was activated by oleoyl imidazolidate (1 mM) at 0°C, 7°C, 25°C, 37°C and 50°C and assayed by the standard procedure.

Fig 38 shows that the speed of the enzyme activation by oleoyl imidazolidate rises with the temperature with 37°C giving the best result. Although activation at 50°C seems to proceed very rapidly in the early stages the enzyme loses activity (related to 37°C) on prolonged incubation. 37°C is therefore the optimum temperature for enzyme activation.

Because the activation of the enzyme proceeds very slowly at 0°C, the acylation reaction may be stopped at any stage by simply placing the activation mixture in an ice bath.

TABLE 5

INCUBATION MIXTURE 37°C

[1- ¹⁴ C] OLEIC ACID mM	[1- ¹⁴ C] OLEOYL IMIDAZOLIDE * mM	INCUBATION TIME min.	a) before c.p.m. extraction	OLEOYL GROUPS IN AQUEOUS PHASE a) μM b) molecules per protein	RECOVERY OF ENZYME ACTIVITY %	ACTIVATION FACTOR
1.4	0	10	4126	0	0	
0	1.0	10 (0°C)	4711	0	0	
0	"	10 (0°C)	"	0	0	
0	"	10	"	0	0	
0	"	10	"	0	0	
0	"	3.5	4348	0.5	> 95%	28.6
0	"	7	"	0.9	"	38.3
0	"	10.15	"	1.1	"	47.7
0	"	21	"	1.8	"	53.3
0	"	10	5057	0.9	"	39

*specific activity 0.21 μCi/μMol

Correlation between radiolabelling and activation of bee venom phospholipase A₂ during incubation with [1-¹⁴C]-oleoyl imidazolidine. Reagents were incubated in standard assay buffer at 37°C and reaction stopped by chilling the solution in an ice bath. Oleoyl imidazolidine and free oleic acid were obtained by shaking the solution with an equal volume (50-250 μl) of cold ethyl acetate following centrifugation at 3000 g. Samples were withdrawn from the aqueous phase for assay (0.5 μl) and ¹⁴C counting (10 μl in 10 ml aquasol).

4.4.3 Protein modification by oleoyl imidazolid

Protein modification has already been studied by lauroyl ethyl carbonate using a gel filtration method to separate the enzyme from the unreacted activator or free acid. The disadvantages of this method were that the activity yields tended to vary and decrease with column useage and it also proved to be unsuitable for rapid quantitative measurements of protein acylation.

A wide range of organic solvents were tested for the extraction of unreacted activator. Only one, ethyl acetate, proved to be adequate for this purpose. As a matter of fact, ethyl acetate extraction proved to be more convenient as a routine method for terminating the reaction and activity yields were quantitative, although the enzyme was less stable after this treatment.

Control experiments with the activator ($[^{14}\text{C}]$ -oleoyl imidazolid) or free $[^{14}\text{C}]$ -oleic acid present in the activation mixture without enzyme, showed that ethyl acetate extracts almost all the activator and the free oleic acid (about 97% of oleic acid was extracted) (Table 5).

Once more, definite proof that the activator attacks the enzyme and does not modify the substrate was obtained by incubating the enzyme with oleoyl imidazolid and extracting all the traces of unreacted activator from the activation mixture using the method that is described above and assaying the purified activated enzyme under the standard procedure. 250 μl of 1 mg/ml bee venom PLA_2 were incubated at

37°C with 1 mM [^{14}C]-oleoyl imidazolide (see Materials and Methods). The reaction was terminated by placing the activation mixture in an ice bath and the unreacted activator was extracted by mixing the activation mixture with equal volume of cold ethyl acetate and centrifuging the mixture at 3,000 g for 5 min. Before and after the treatment with ethyl acetate, equal volume samples were withdrawn and counted for radioactivity by placing the samples in vials containing 10 ml aquasol (Table 5). After the centrifugation, 0.5 μl samples were withdrawn from the protein phase (aqueous phase) and assayed by the standard procedure to test whether the enzyme is still activated after the treatment and also to check for the recovered activity (Table 5).

Experiments in which the activation of the enzyme was terminated at different times during the incubation showed that a 47-fold activation corresponds to the addition of a single acyl group per protein molecule (Table 5). Excess oleoyl residues tended to bind during the reaction period required for complete activation (53-fold) (Table 5).

Gel electrophoresis experiments in which the activated and control enzymes were run under different conditions, for example, isoelectric focussing, cathode-running gel systems and SDS PAGE system, failed to resolve these two forms of the enzyme.

These negative results suggest that the acylated residue of the activated enzyme is a tyrosine, because tyrosine has a very high pK, and also imidazolides have been used by other workers as

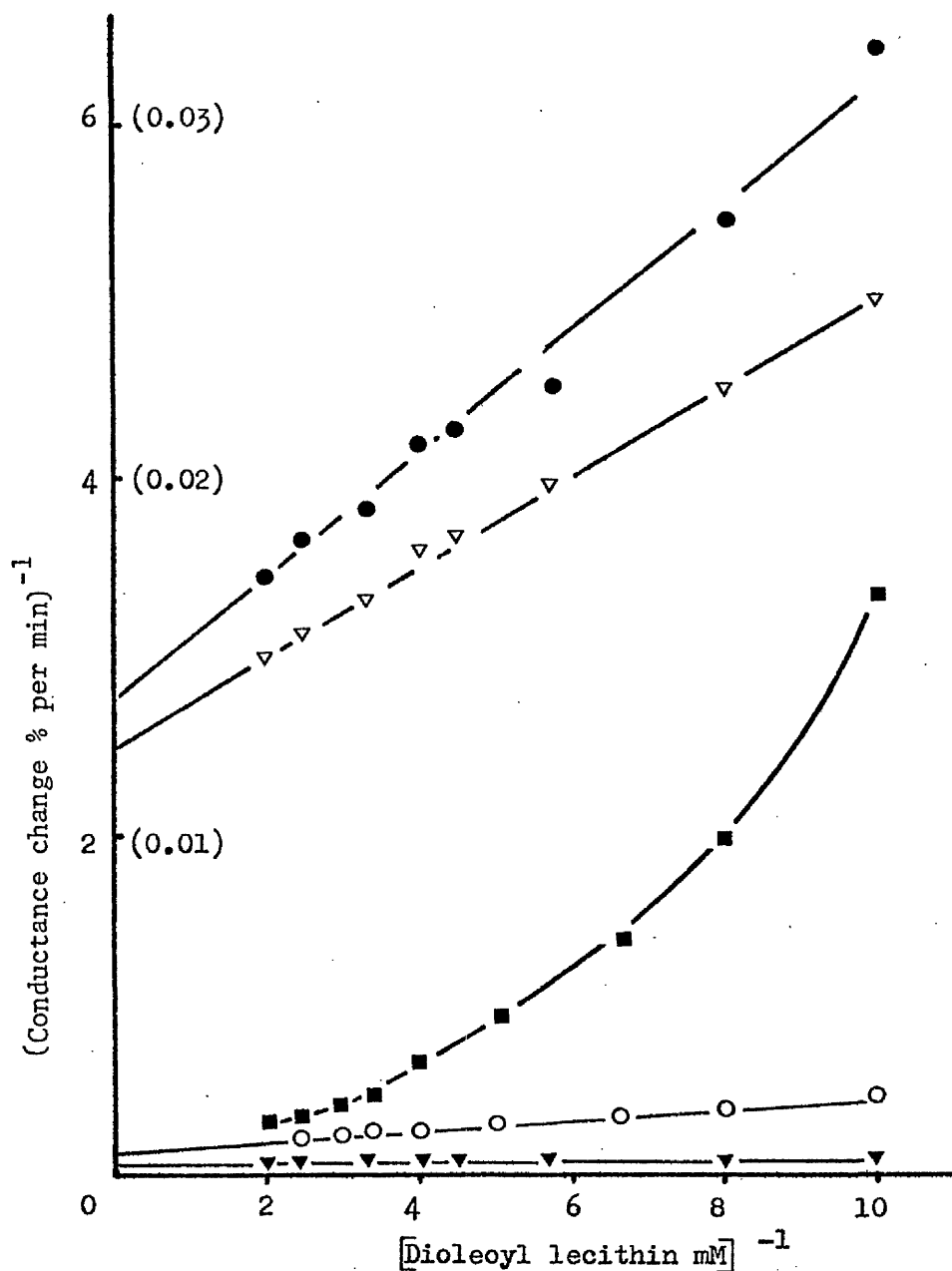


Fig (39) Double-reciprocal plot of the activity of Bee venom PLA_2 towards dioleoyl lecithin.

● initial rates and ■ maximum rates for untreated enzyme,
 $V_{\max} = 64$ mol per min. per mg protein, $K_m = 0.13$ mM .

▼ initial rates for enzyme preactivated with oleoyl
imidazolid (see Fig 36), ▽ data for preactivated enzyme
replotted with 50-fold increased ordinate scale,

$V_{\max} = 3,500$ mol per min. per mg protein = 816 moles per
sec. per mole of protein, $K_m = 0.10$ mM. ○ initial rates for
enzyme in the presence of 0.17mM oleic acid. All rates were
measured under the standard assay conditions at 37°C .

moderately specific reagents for tyrosine (Simpson et al, 1963). However, efforts to reverse activation by incubating the activated enzyme with 1M hydroxylamine at pH 8.0, following the method described by Riordan et al (1965), slowly inactivated the enzyme but did not restore the biphasic character of the hydrolysis curve of dioleoyl lecithin.

4.4.4 Steady state Kinetics

Because the enzyme does not act on a true solution of substrate it was not expected that it would conform to classical steady state kinetic behaviour.

The kinetic behaviour of bee venom phospholipase A_2 was studied against dioleoyl phosphatidyl choline substrate in the 20% propanol system. Because of the biphasic character of the hydrolysis curve of dioleoyl lecithin by the bee venom PLA_2 , the initial and late rates were plotted separately against substrate concentration and a Lineweaver-Burk double reciprocal plot of the data was made. Fig 39 shows that initial rates follow normal kinetic behaviour, in contrast late rates (maximum product activated rates) shows a sigmoidal double reciprocal plot because at high substrate concentration, product activation is well defined whilst at low concentration it vanishes.

Kinetic analysis of enzyme activated by oleoyl imidazolidine shows that activation is almost entirely determined by increase of the V-max term (53-fold) with a small favourable modification of K_m (0.13 mM - 0.10 mM). The enzyme was activated by oleoyl imidazolidine

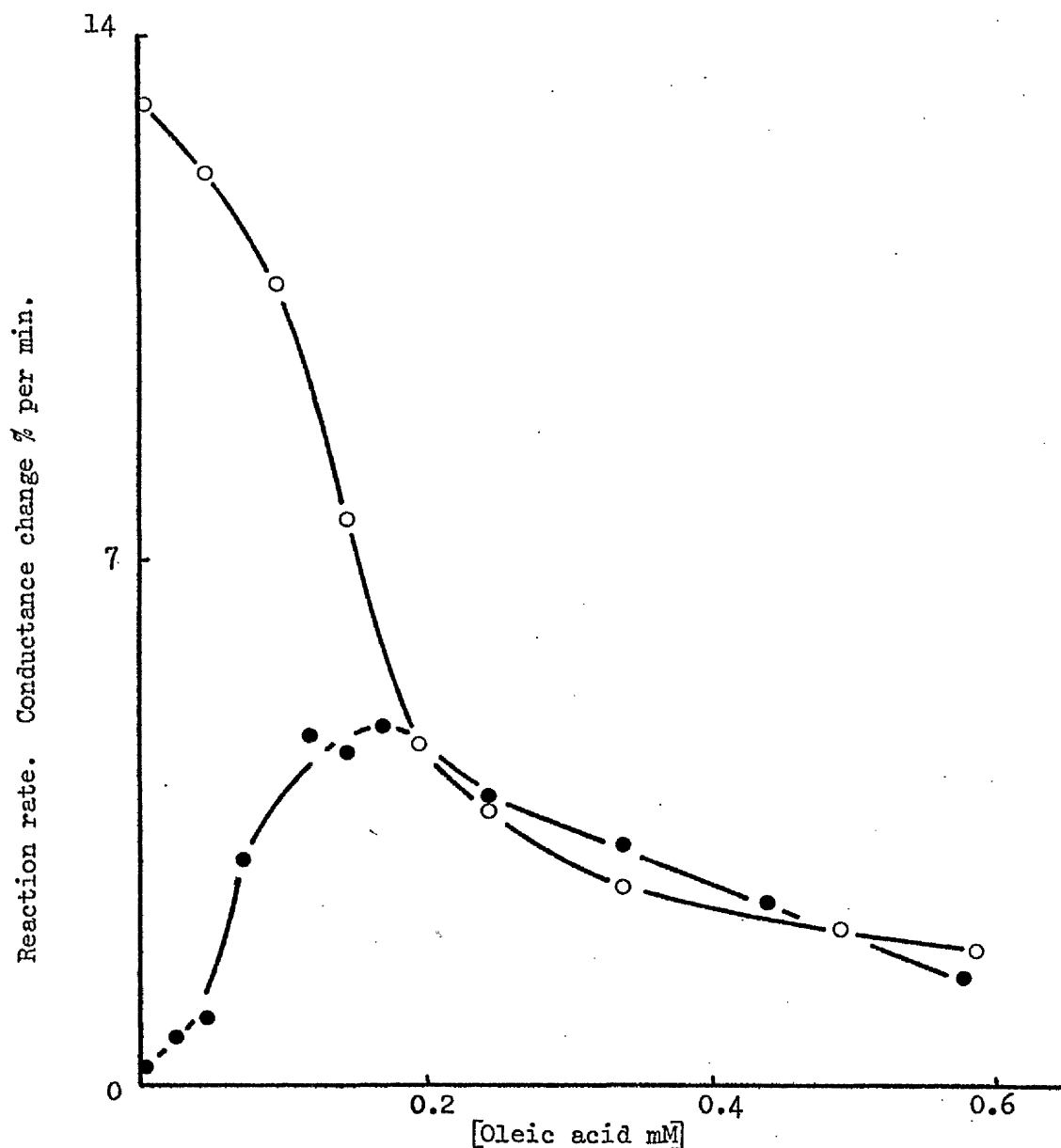


Fig (40) The effect of oleic acid on bee venom PLA_2 and on enzyme pre-activated with oleoyl-imidazolide.

The hydrolysis of dioleoyl phosphatidyl choline (0.34 mM) by bee venom PLA_2 (2.5 μg per ml) was followed by the standard assay conditions at 37°C and the initial rates plotted against oleic acid concentration.

● untreated enzyme, ○ enzyme (1 mg/ml)
pre-activated with 1 mM oleoylimidazolide (see Fig 36).

by the method described above, and assayed under the same conditions as the control enzyme, and the reciprocal of the initial rates were plotted against the reciprocal of the substrate concentration (Fig 39).

Enzyme activated by an optimal concentration (0.17 mM) of free oleic acid shows similar kinetic behaviour with V-max determined activation. Assay conditions were the same as the conditions described for the control enzyme and the reciprocal of the initial rates were plotted against the reciprocal of the substrate concentration (Fig 39).

Efforts to find out whether the enzyme, maximally activated by free oleic acid, was as active as that activated by oleoyl imidazolidide failed, because oleic acid was inhibitory both to the control and the covalently activated enzyme (Fig 40). This inhibition seems to be competitive and is reduced at high substrate concentrations. These results (Fig 40) suggest that activation by free acid and by the bound oleoyl residue may produce similar changes in the catalytic activity. This also shows that under experimental conditions the acylating agents are far more effective activators than the free acids.

4.4.5 Calcium dependence

Calcium is the natural activator for the bee venom PLA₂ (Shipolini et al, 1971) and the enzyme is inactive in the absence of calcium. In the presence of excess EDTA both the control and the oleoyl imidazolidide-activated enzyme are completely inhibited.

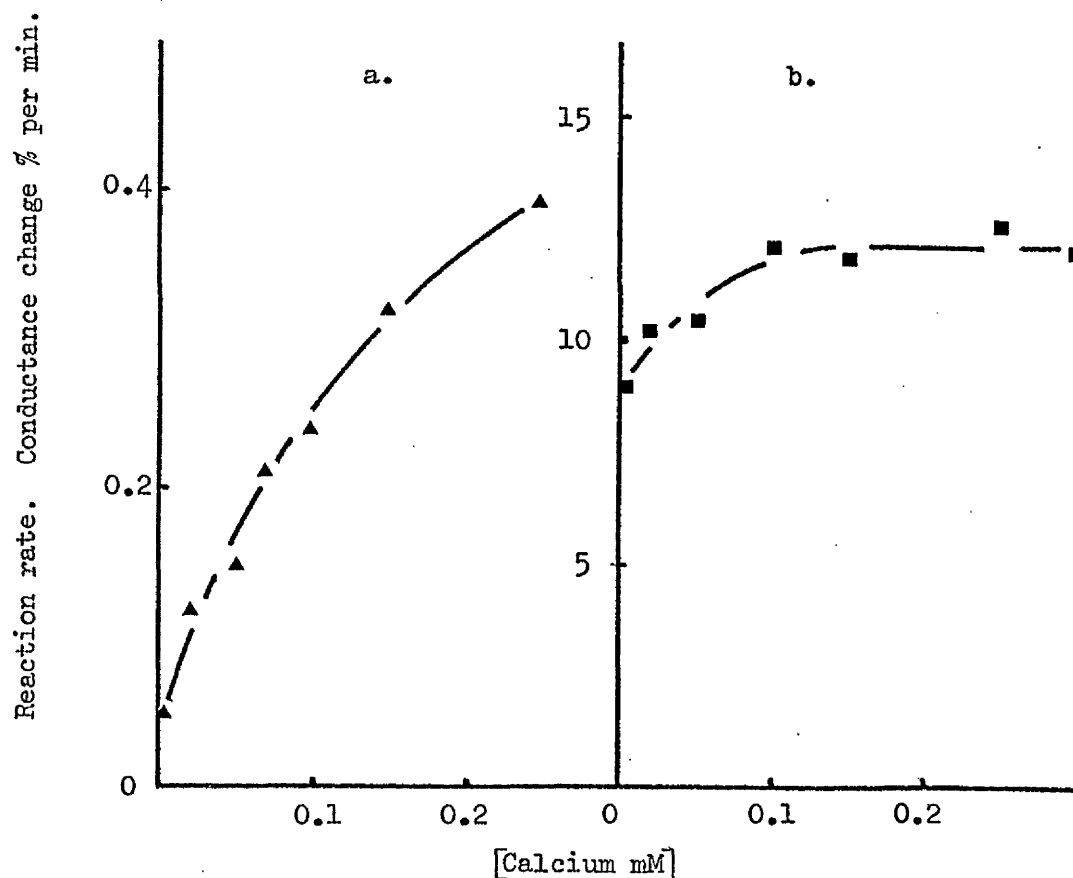


Fig (41) The effect of calcium on untreated and pre-activated Bee venom PLA₂.

Initial rates of hydrolysis of dioleoyl lecithin (0.24 mM) by PLA₂ (2.5 µg/ml) plotted against Ca²⁺ concentration, measured under the standard assay conditions at 37°C.

a) untreated enzyme

b) enzyme (1 mg/ml) preincubated with 0.6 mM oleoyl imidazolidine for 20 min at 37°C and assayed at an enzyme concentration of 0.5 µg/ml.

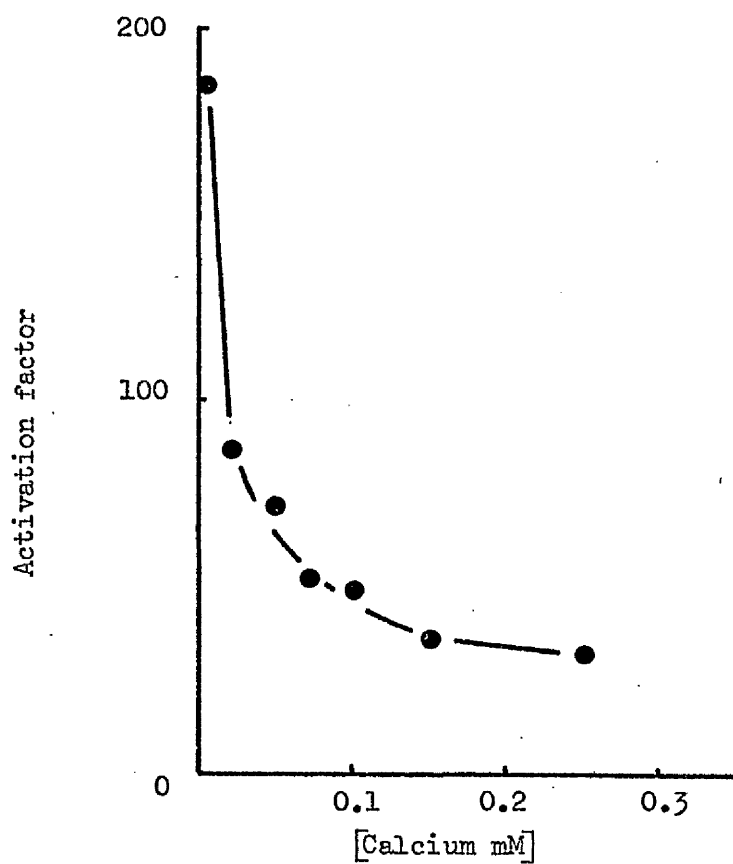


Fig (42) The degree of activation of the Bee venom PLA₂ by oleoyl imidazolidine plotted against calcium concentration.

The activation factor is expressed as the ratio of initial reaction rates of the Fig (41 a,b).

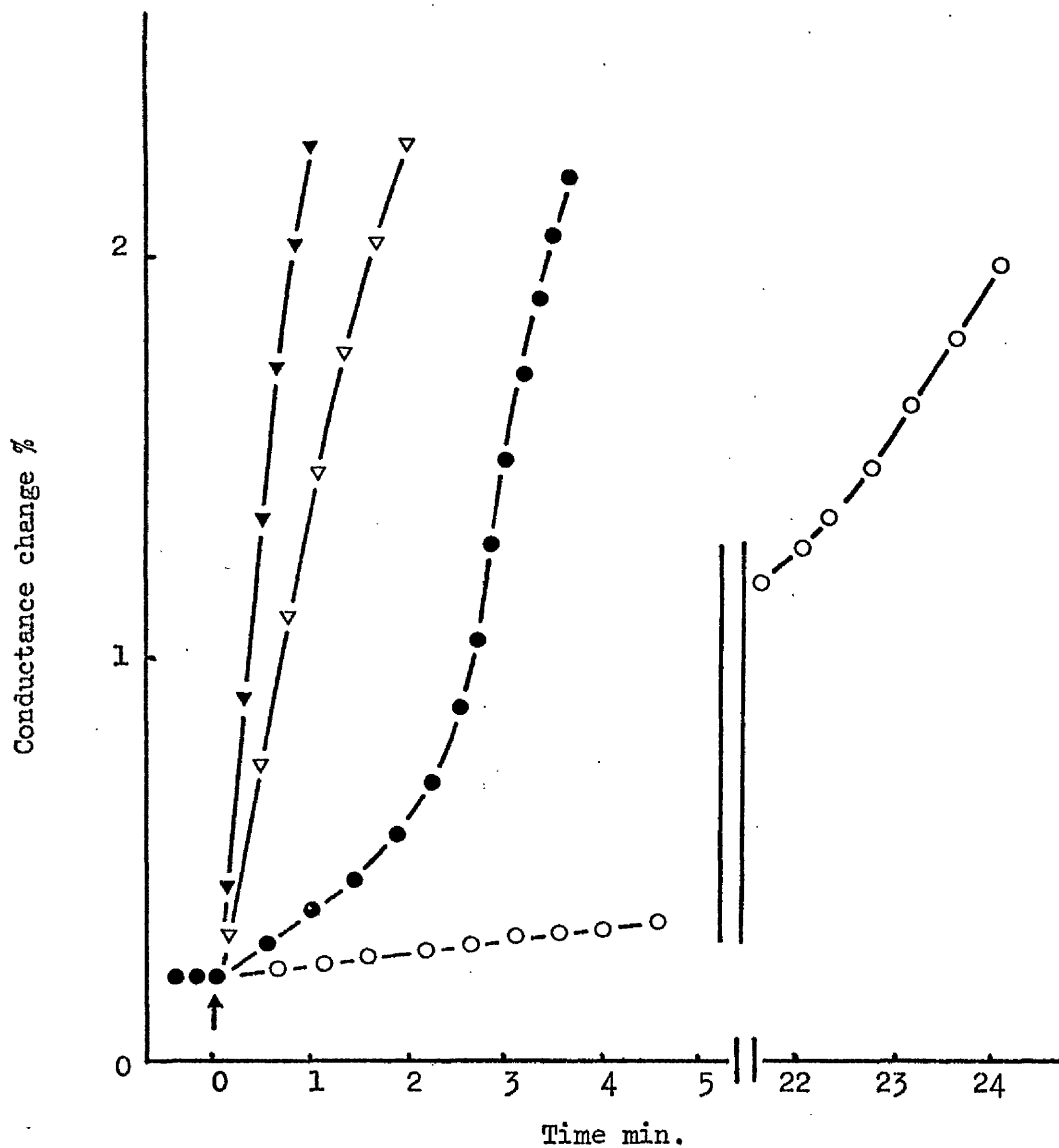


Fig (43) The effect of strontium on bee venom PLA₂ and on enzyme preactivated with oleoyl imidazolid.

The hydrolysis of dioleoyl lecithin (0.24 mM) by bee venom PLA₂ (2.5 µg per ml) at 37°C measured under standard assay conditions and in the presence of 0.1 mM SrCl₂.

- control enzyme 0.1 mM CaCl₂,
- control enzyme 0.1 mM SrCl₂,
- ▼ preactivated enzyme 0.1 mM CaCl₂,
- ▽ preactivated enzyme 0.1 mM SrCl₂.

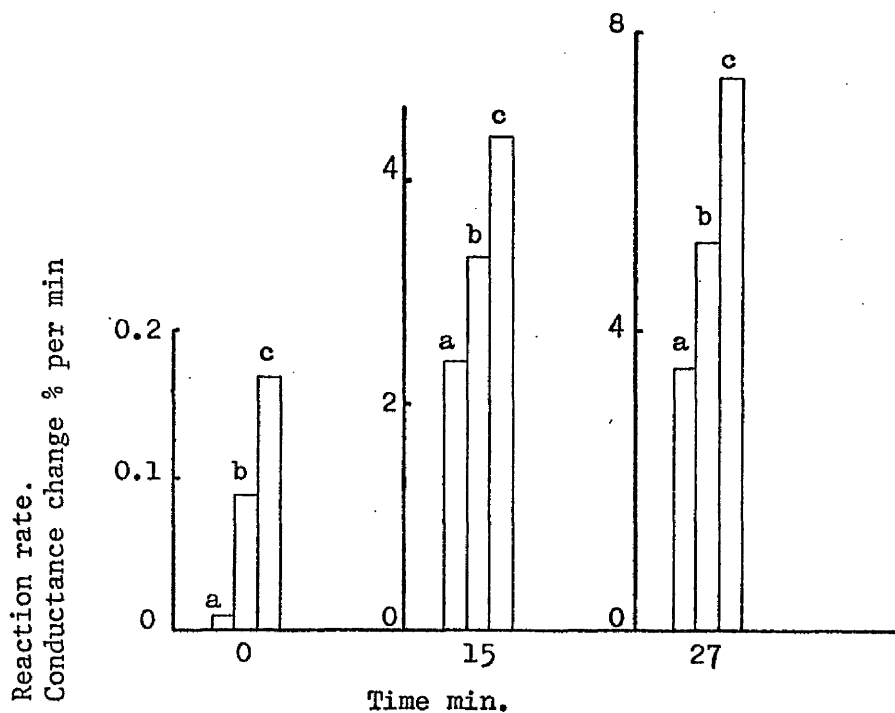


Fig (44) The effect of strontium on the activation of Bee venom PLA₂ by oleoyl imidazolide.

The enzyme (1mg/ml.) was incubated with 1mM oleoyl imidazolide at 37°C in propanolic buffer in the absence of calcium and 2.5 µl samples withdrawn for assay at measured time intervals.

The initial rates of the hydrolysis of dioleoyl lecithin (0.24mM) by bee venom PLA₂ (2.5µg/ml.) measured by the standard method at 37°C. a, in 0.1mM Sr²⁺. b, in the absence of Ca²⁺. c, in 0.1mM Ca²⁺.

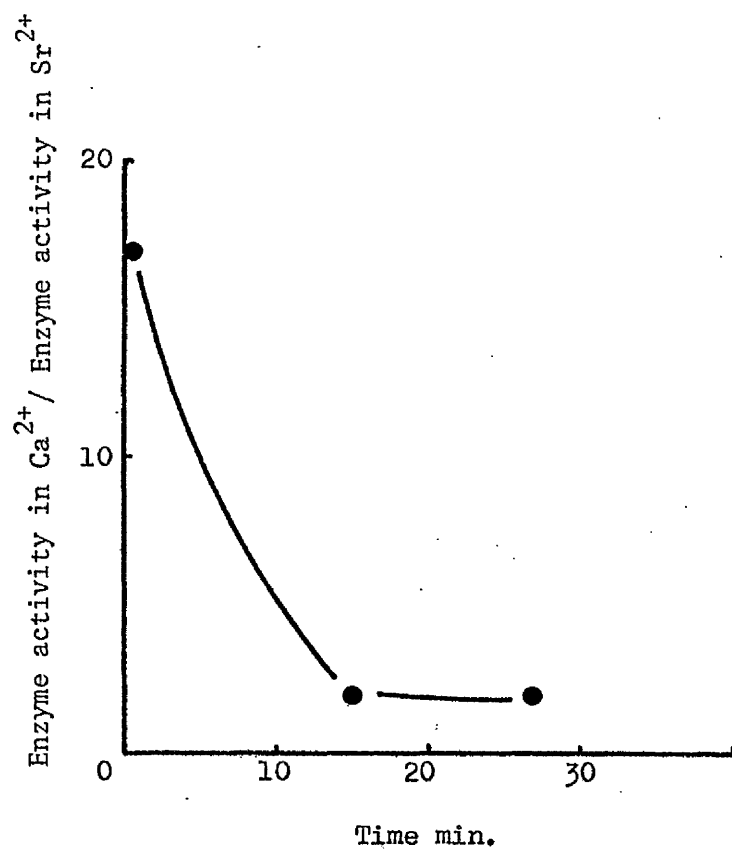


Fig (45) The inhibition of Bee venom PLA_2 by strontium during the enzyme activation by oleoyl imidazolide. The inhibition is expressed as the quotient of the enzyme activity in Ca^{2+} over the enzyme activity in Sr^{2+} during the enzyme activation shown in Fig 44.

Concentration dependence curves for the calcium requirement of both control and activated enzyme showed that the activated enzyme requires lower calcium concentrations than the control enzyme for activity (Fig 41 a,b). Fig 41a shows that additional calcium over that is always present in the control enzyme samples is activating. In contrast to the control enzyme the oleoyl imidazolid activated enzyme was almost insensitive to excess calcium (Fig 41b). Comparing the activation level for both control and activated enzyme, the maximum activation factors are found at the lowest calcium levels consistent with catalytic function of each protein molecule (Fig 42).

These results suggest that the enzyme might have two sites for calcium. One calcium appears to be strongly bound, (based on the fact that enzyme has high activity in the absence of added calcium, which can be abolished by EDTA) and one weakly bound which gives $\frac{1}{2}$ maximum activation at $10^{-4} \text{ M Ca}^{2+}$.

In experiments where calcium was replaced by strontium, a competitive inhibitor, both control and oleoyl imidazolid activated enzyme showed some inhibition, but the activated enzyme was much less inhibited than the control enzyme (Fig 43). Fig 44 shows the activation of bee venom PLA_2 assayed in 0.1 mM Sr^{2+} , in the absence of Ca^{2+} and in 0.1 mM Ca^{2+} , the enzyme was activated in the absence of Ca^{2+} . In all cases the assay mixture was 1 ml 20% propanol, 10 mM triethanolamine/HCl pH 8.2 with 0.24 mM dioleoyl lecithin. These results show that activation of the enzyme reduces its sensitivity to activation by Ca^{2+} (Fig 42) and inhibition by Sr^{2+} (Fig 45). This

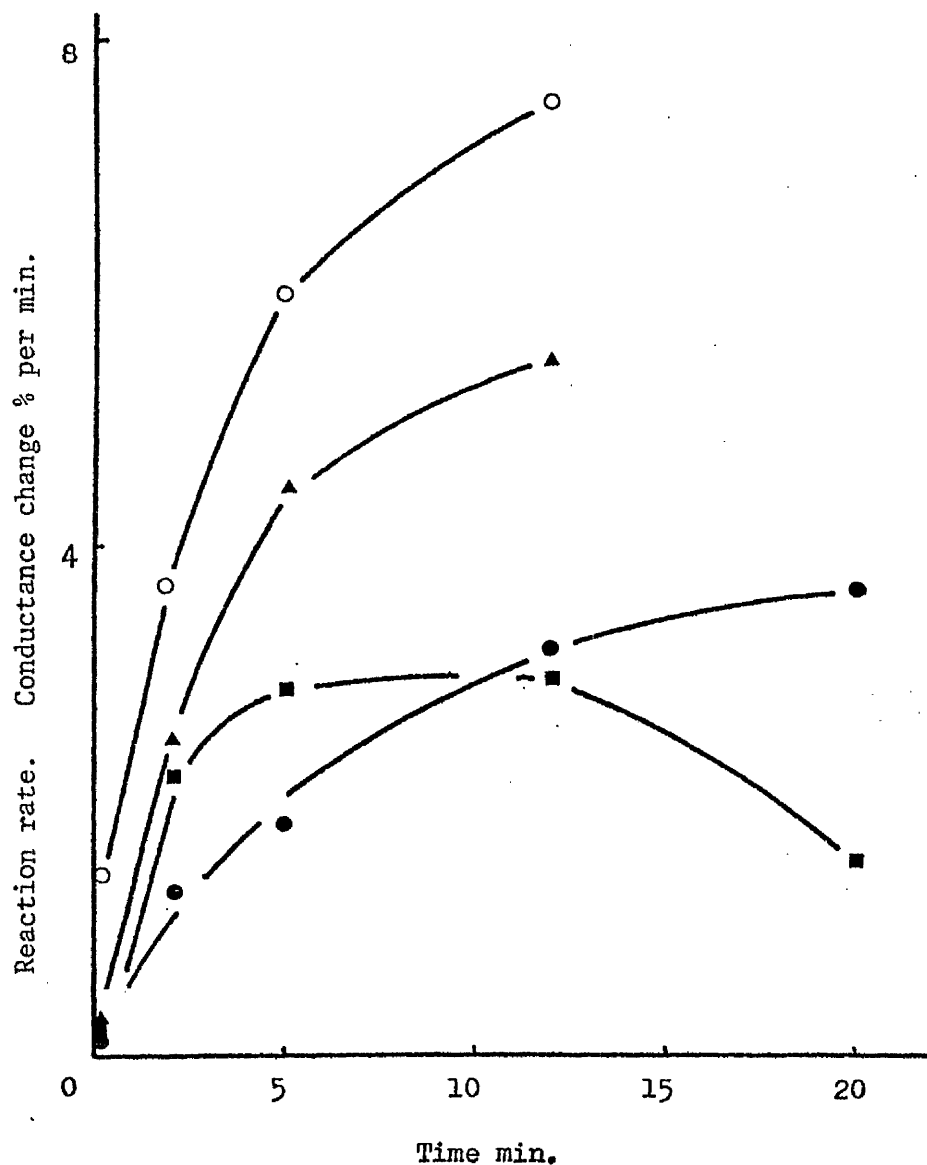


Fig (46) The role of calcium in the activation of Bee venom PLA₂.

The enzyme (1mg/ml.) was incubated with 1 mM oleoyl imidazolide at 37°C in Ca²⁺ free propanolic buffer, with 0.1mM CaCl₂ ▲ , with 0.3mM CaCl₂ ○ , with 0.2mM EDTA ■ , with 0.2mM EDTA and 2.4mM dioleoyl lecithin ●

The initial rates of the hydrolysis of dioleoyl lecithin (0.24mM) by bee venom PLA₂ (2.5 μg/ml.) at 37°C measured under the standard procedure.

is consistent with the idea that both Ca^{2+} and Sr^{2+} bind at the same site of the protein; fatty acid-type activation overrides the action of metals at these sites. These results suggest that fully activated enzyme might be completely insensitive to Sr^{2+} and also insensitive to second weakly interacting calcium ion.

In experiments where the enzyme activation was carried out at different calcium concentrations, the additional calcium stimulates the enzyme activation and a complete depletion of calcium changes the response to activation in two ways; the rate of activation is lowered and the enzyme shows clear signs of progressive inactivation (Fig 46); (all traces of calcium were removed from the activation mixture by addition of excess EDTA). In the complete absence of Ca^{2+} the catalytic activity of the enzyme is abolished, so it is possible to include substrate in the activation mixture to test its action on activation. When this was done it was found to protect the enzyme against the inhibition seen in the absence of calcium (Fig 46).

Chapter V : Activation of bee venom phospholipase A₂
in aqueous systems

Although the activation of bee venom PLA₂ in the 20% n-propanol system gave dramatic results, this system has some disadvantages; the presence of the organic solvent (n-propanol) slows down the speed of action of the enzyme and under these conditions the substrate structures are not well characterized. Glycerophospholipids do however form well defined structures (micelles or liposomes) in aqueous medium. Fatty acid activation cannot be seen in the aqueous system suggesting that it might be an artefact of the n-propanol medium or that fatty acids interact with calcium to lower the effective concentration of both activators. Therefore it was very important to test whether or not activation can be achieved in the aqueous system.

The action of bee venom PLA₂ towards short and long-chain lecithins is described in the chapter III.

The enzyme activated by oleoyl imidazolid shows little or no enhancement of activity with short chain lecithins ($R_1, R_2 < C_{12}$ or $R_2 < C_9$); compounds which form micelles in water. In contrast with the micellar substrates the acylated enzyme shows considerable activation with liposomal substrates (long chain lecithins). Unfortunately long chain fatty acids ($R > C_{12}$) bind calcium, a necessary activator of the enzyme, very rapidly under these conditions, therefore the conductimetric assay can not be used. To overcome this problem substrates were synthesized which had short acyl chains in the 2-position and long acyl chains in the 1-position, these were conveniently prepared from egg lysolecithin. These compounds form

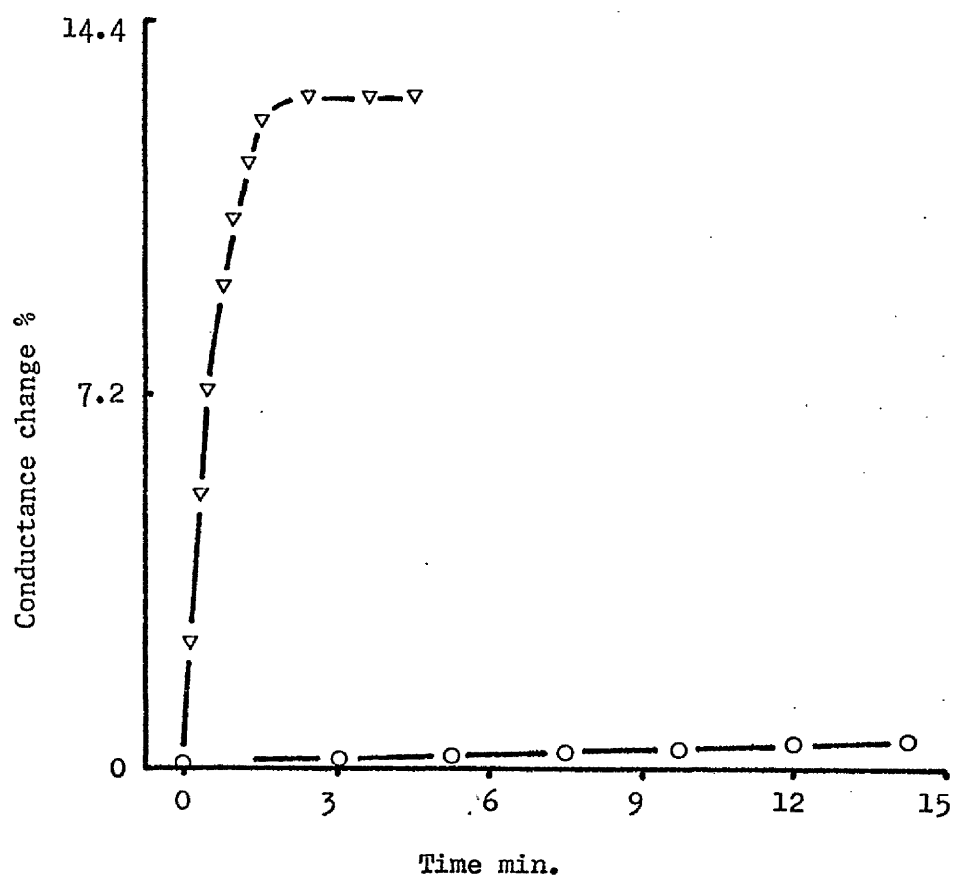


Fig (47) Activity of untreated and preactivated Bee venom PLA₂ in an aqueous buffer system.

The hydrolysis of 2-lauroyl lecithin by PLA₂ (5μg/ml.) at 37°C followed by conductimetric assay in 1ml. 10mM triethanolamine/HCl buffer, pH 8.2 containing 0.1mM CaCl₂.

O untreated enzyme, ∇ enzyme (2mg/ml.), preincubated with 0.5mM oleoyl imidazolide.

liposomes in water and the fatty acid which is liberated by the phospholipase A₂ action does not bind calcium at low concentration, so that the complete course of the reaction can be followed under the experimental conditions.

100 µl of 2 mg/ml bee venom PLA₂ prepared in aqueous buffer (10 mM triethanolamine/HCl, pH 8.2, containing 0.1 mM CaCl₂) were incubated with 0.5 mM oleoyl imidazolide and 2.5 µl samples were withdrawn and assayed in 1 ml 10 mM triethanolamine-HCl pH 8.2 containing 0.1 mM CaCl₂ and 2-lauroyl lecithin liposomal substrate. Fig 47 shows that the activated enzyme abolishes the lag phase of the reaction.

In order to demonstrate activation of the enzyme in the aqueous system it was necessary to use a much lower concentration of the activator (at least 4-fold less) than the standard (1 mM). When the enzyme was activated by the standard concentration of oleoyl imidazolide, and assayed in the aqueous system, inhibition of the enzyme towards the liposomal substrate was apparent. Controls where the enzyme was activated by oleoyl imidazolide (standard concentration) in propanolic or aqueous buffer and assayed in the 20% propanol system, showed the activation of the enzyme was similar for both cases. The difference between the two systems was due to the assay mixture and not to the activation mixture.

These results suggest that the mechanism of the enzyme activation in the aqueous system might be different from the 20% propanol system. Two explanations can be given, either the enzyme

requires strictly one acyl residue per protein molecule to activate, and all the additional residues are inhibitory, or the enzyme acts as a dimer (Shipolini et al, 1971 first suggested that the bee venom PLA₂ might exist as a dimer). It is probable that only one acyl residue would be required per dimer (two protein molecules) and the additional residue could be inhibitory because it might prevent the dimerization of the enzyme.

Chapter VI : The action of activated bee venom PLA_2
on Red Blood Cells

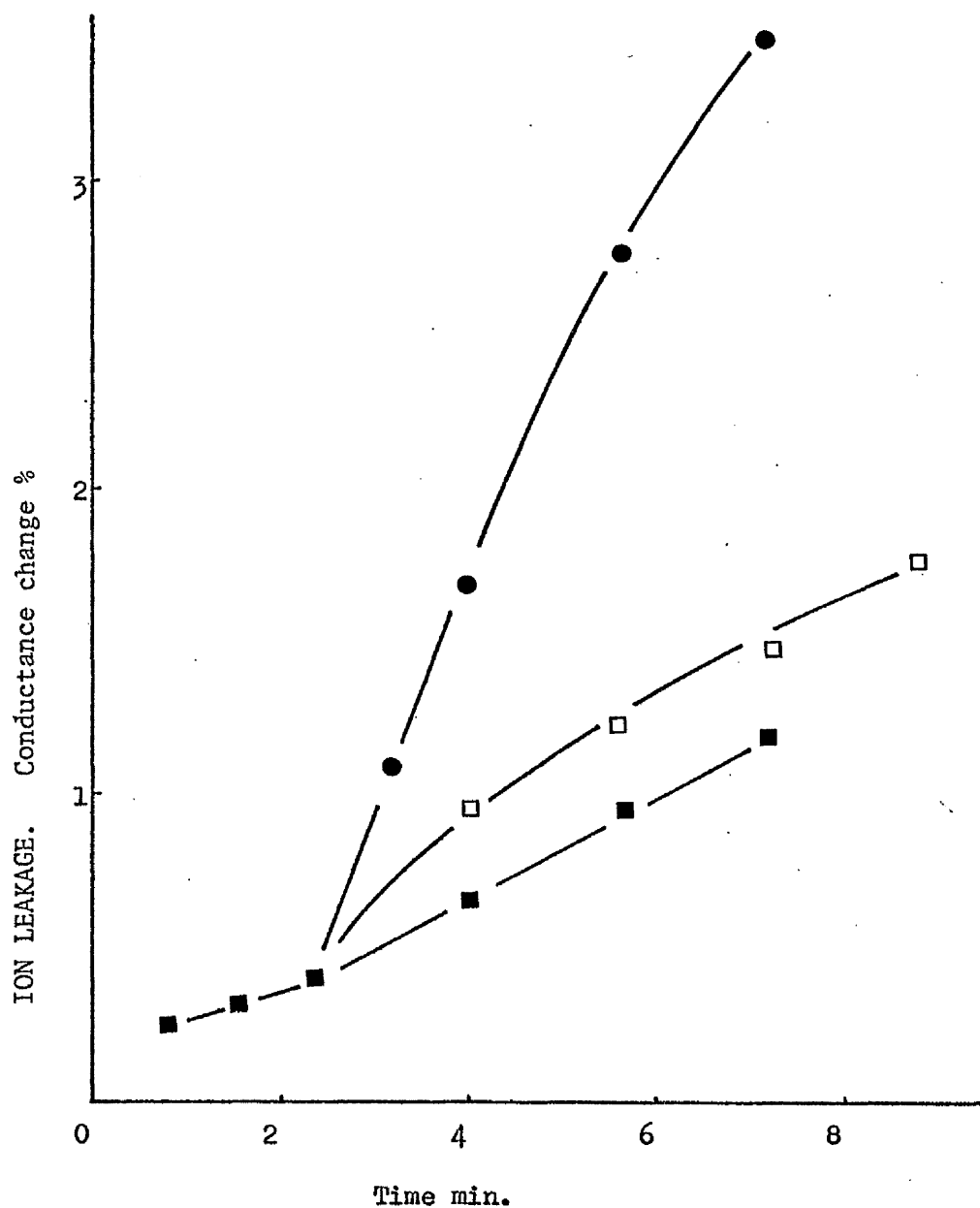


Fig (48) The effect of activated Bee venom PLA_2 on rabbit erythrocytes.

The enzyme (2mg/ml.) was incubated with oleoyl imidazolide (0.2mM) and 1 μ l samples were applied to 1ml. isotonic sucrose solution as described in the text.

■ control, ● enzyme preactivated with oleoyl imidazolide, □ preactivated enzyme plus 1 μ g lysolecithin.

Bee venom PLA₂ has no lytic or prelytic action on rabbit erythrocytes unless sublytic levels of exogenous long chain fatty acids (e.g. oleic, linoleic) are also present (Lawrence et al, 1974). This synergetic lytic action of the bee venom enzyme can be inhibited by low levels of lysolecithin (Lawrence, 1975). However, addition of free long chain fatty acids or lysolecithin to erythrocytes suspension, without the presence of phospholipase, have lytic or prelytic action (Lawrence et al, 1974).

Bee venom PLA₂ preactivated with oleoyl imidazolide causes prelytic damage in rabbit erythrocytes and this effect is inhibited by low levels of lysolecithin (Fig 48). 100 µl of 2 mg/ml bee venom PLA₂ prepared in aqueous 10 mM triethanolamine/HCl buffer pH 8.2 containing 0.1 mM CaCl₂ were incubated with stoichiometric amount of oleoyl imidazolide at 37°C and 1 µl samples were applied to 1 ml isotonic sucrose solution buffered with 10 mM MOPS/Na⁺, pH 7.5 at 37°C containing rabbit erythrocytes suspension. Leakage of electrolyte from erythrocytes was followed by conductimetry (Fig 48).

The effect of activated enzyme is by no means as large as that of enzyme in the presence of free fatty acid and the activated enzyme gives a larger response in the presence of free fatty acid than does the control enzyme.

These results suggest that fatty acids can act by two means in this system; they activate the enzyme directly, and also modify the physico-chemical properties of the substrate.

Because melittin (the main toxin of bee venom) causes lytic damage to erythrocytes, it was necessary to test whether or not it stimulates the action of the phospholipase A_2 towards rabbit erythrocyte membranes. High levels of melittin lyse the erythrocytes spontaneously. Rabbit erythrocytes were incubated with sublytic levels of melittin and then the control or the oleoyl imidazolide activated enzyme was added. The results showed that melittin weakly activates both the control and the activated enzyme. When melittin was tested for activation by oleoyl imidazolide, a slight inhibition was observed.

These results show clearly that oleoyl imidazolide modifies only the enzyme phospholipase A_2 and not the peptide melittin which might be present in the enzyme preparations.

Chapter VII : Discussion

7.1 Conductimetry

The present work uses conductimetric assay techniques almost exclusively. This must be emphasised because no other workers in the field have published results with this method. It is not easy to discuss why conductivity has been ignored by other workers as the advantages it possesses for this type of work are very numerous. The results of this study are rather different from these obtained by other groups and it is necessary to show that it is due to specific advantages of the method and not due to experimental artefacts.

Product activation of bee venom PLA₂ is detectable only in the complete absence of free fatty acids in substrate preparations and both synthetic and purified natural phospholipids tend to be contaminated with long chain fatty acids. The great advantage of conductimetry over other methods is that very small amounts of free fatty acids can be detected. Zwitterionic substrates like choline glycerophospholipids are not conducting molecules, because of this the presence of free fatty acids cannot be overlooked by conductimetry, while with any other method there is no immediate indication of their presence. Therefore all the substrates were highly purified and were free from fatty acids. The failure of other workers to observe fatty acid activation of phospholipase: A enzymes may also due to other aspects of their kinetic methods. For example, pH stat titration, the most common method for this type of work, has a big disadvantage in that it cannot record rapid changes in rates which may occur during the course of the reaction. pH electrodes have a rather slow response speed ($T_{\frac{1}{2}}$ ca. 1 sec.), but also the electro-mechanical linkages, including stirring, limit

response speed still further. The main problem is however that the pump speed which gives best tracking of a given reaction rate is not suitable for other rates. This work will also show that non-physiological substrates, for example, the glycol analogues used in sensitive spectrophotometric assays, could not reveal fatty acid activation.

Because of the six conductivity cells which all can be recorded at the same time, extensive kinetic results can be produced within a short period of time. Also, due to the small size of the conductivity cells reaction mixtures reach thermal equilibrium rapidly (within 1-2 min), so there is no delay between sets of assays. With the high stirring efficiency, events occurring within seconds may be accurately recorded (note that thermal equilibration is not important for small additions to the reaction mixture). This means that the initial rates and any rapid change of rate that may occur during the course of the reaction can be recorded with high accuracy. Furthermore, the development of the new conductivity cell and the modifications to the electronic part of the apparatus (see Materials and Methods chapter), gave the system a stable response at very high sensitivities. This meant that conductance changes as small as 0.01% now could be resolved easily. Hence very low enzyme or substrate concentrations can be used and also enzymes with low activity can be detected.

Conductivity more than most other physico-chemical methods, responds to changes due to interactions of all components of the system. Small changes which may have no effect on real reaction rates might produce unforeseen changes in the response. This is very

important in the present study, as the interactions of fatty acids, calcium, substrate, counter ions are all influenced by temperature and solvent concentration and it is important to calibrate and note the effect of all possible variables.

The best example of an experimental artefact in the reaction occurs when long chain substrates are hydrolysed in propanolic media. Calibration by addition of fatty acid gives linearity at reduced propanol concentrations in the absence of substrate. However in the presence of substrate the conditions for linearity are critical and the range of solvent concentration is restricted. Thus at low solvent levels the reaction time course shows a lag phase which is largely due to substrate product interaction and not product activation. Furthermore interactions between calcium and long chain fatty acids might produce the same effect at reduced solvent concentrations or even prevent any reaction rates from being measured. Calcium precipitation by long chain fatty acids was the main problem of the phospholipase assays in aqueous systems. To overcome this problem, excess calcium or short chain substrates have been used for the aqueous system.

There is no doubt that conductimetry is a convenient, rapid and simple system and the results obtained in the present work are by no means due to artefacts.

7.2 The action of Bee venom phospholipase A₂ on Lecithin

Bee venom PLA₂ shows different action towards choline glycerophospholipids with different acyl chains due to the type of

structure that these compounds form in water and in dilute aqueous solutions of organic solvents. Generally the enzyme shows product activation towards long-chain choline glycerophospholipids in both 20% propanol and aqueous systems due to the long chain fatty acids or lysolecithin respectively. Although the aqueous assay provides the most natural system for studying the action of the enzyme, propanolic assay system has been mainly used. The reason for this is that long chain fatty acids bind calcium, the natural activator of the bee venom PLA_2 , very strongly in the aqueous assay system, so it was practically impossible to follow the hydrolysis of the long-chain lecithins by the bee venom enzyme with conductimetry.

In theory the falling conductivity at high calcium concentration in the presence of an anionic buffer (e.g. Bicine) in an aqueous medium gives equal sensitivity to the rising conductivity at low calcium, a propanolic medium and a cationic buffer (e.g. tri-ethanolamine). The latter is technically more satisfactory. The principal reason for this is that the substrate is apparently soluble in the propanolic medium but insoluble in water and this gives unsteady basal conductance. Furthermore the interaction between fatty acid and calcium may not be rapid at low product levels and reaction rates are difficult to interpret. A rather more important problem is that non-ionic detergents are product activators in the aqueous system but have little effect in the propanolic medium.

n-Propanol is the best solvent for this purpose because it is a non-volatile solvent, there is no calcium precipitation under the experimental conditions (20% n-propanol-water assay system) and also the

solvent acts as a detergent for the electrodes of the conductivity cells. The main disadvantage of this type of assay system is that glycerophospholipids form poorly characterized structures in the presence of the organic solvent, and it is probable that the intrinsic activity of bee venom PLA₂ is lower in the presence of organic solvents.

After the best assay conditions for the bee venom PLA₂ towards long-chain lecithin had been established, it was important to use a well characterized substrate but one which shows the same behaviour as natural lecithins under these assay conditions. Dioleoyl lecithin was chosen for several reasons. It is structurally close to the substrates found in membranes where 1-palmitoyl,2-oleoyl phosphatidylcholine might be the most abundant, it is readily synthesized, soluble in the propanolic medium and easy to disperse in the aqueous medium.

7.3 The activation of Bee venom phospholipase A₂ by free long-chain fatty acids

Early workers, principally Dawson and his collaborators, established that the activation of several phospholipases by amphipathic compounds, including fatty acids, was due to the change of the ζ -potential (net charge) of the substrate surface by these compounds. Similar conclusions have been reached by many workers. Three possible explanations for the substrate mediated mechanism can be given:

- a) these compounds might increase the attraction between enzyme and substrate, b) they might increase the penetration ability of the enzyme in the interface, c) the substrate modified by these compounds might force allosteric changes in the enzyme configuration.

The present results show that the activation of bee venom PLA₂ by free long chain fatty acids is dependent on the chain length and the degree of unsaturation of the fatty acids with oleic acid the most effective. This pattern of activation is similar to that found by Epstein and Shapiro (1959) and Waite et al (1969).

The theories of substrate mediated activation all require that the activator is intercalated by the substrate, thus it would be more probable with long chain substrates and long chain acid activators. In addition activation for a given substrate and a given acid should be determined by the ratio of acid to substrate and not by the absolute concentration of acid. This is quite consistent with the results of Epstein and Shapiro (1959) who reported that the intestinal mucosa phospholipase A had optimum activity when the substrate and fatty acids were in equimolar quantities. This is in complete disagreement with the present results where the activation is not dependent on the relative concentration of the acids and substrate.

The major alternative to a substrate-mediated mechanism for phospholipase activation is allosteric modification of the enzyme. This is supported by the fact that conductimetric analysis showed little interaction between fatty acid and the substrate. These doubts that the substrate-mediated mechanism might not apply to all the phospholipases, combined with Dawson's results (1963) which did not support such a mechanism for the cobra venom (Naja-Naja) phospholipase A₂ and the results published by Verger et al (1973) that the pancreatic PLA₂ does not require a negative ζ -potential at the

interphase in order to degrade lecithin (because incorporation of short chain lecithin molecules into the organized aggregates of long chain lecithin has the same effect), were sufficient to encourage further investigation for the mechanism of the activation of bee venom PLA₂.

7.4 The activation of Bee venom phospholipase A₂ by acylating agents

Because activation by free long chain fatty acids did not provide sufficient evidence to elucidate the mechanism of the activation acylating agents have been used to avoid the problems which arise when free acids are present in the system. Taking as a control the hydrolysis curve of the dioleoyl lecithin by bee venom PLA₂ (Fig 17) activation of the enzyme was characterized by two criteria; the unimodal slope of the hydrolysis curve and the degree of activation.

Lawrence and Moores (1975) had demonstrated an irreversible activation of bee venom PLA₂ when the enzyme was treated by decanoic anhydride. Unfortunately activation by anhydrides did not satisfy the above criteria, for example, enzyme activated by anhydrides did not give unimodal slopes.

Although lauroyl ethyl carbonate did not produce unimodal curves it gave much higher activity levels than the anhydrides and many useful results were produced using this compound as an activator. Definite proof that the activator works by modifying the enzyme was obtained with gel filtration studies using [¹⁴C]-labelled LEC. The enzyme retains full activation after the treatment and is stable in

high activity form indefinitely. These results established without any doubt that bee venom PLA₂ activation by acylating agents follows an enzyme-mediated mechanism. The results were not, however, good enough to justify the use of the reagent in studies of the chemistry of the activation site. This reagent turned out to be relatively specific, but it was quite clear that uptake of 1.8 lauroyl residues did not give full activation and the further treatment was inhibitory. However the activation site of the protein seems to be more reactive than any other site towards this reagent.

In order to understand the chemistry of the activation it was necessary to have a high specific activator which would follow the criteria stated above. Fortunately such a compound was finally found. Long chain fatty acid imidazolides proved to be the best activators tested, with oleoyl imidazolidide giving the most satisfactory results. This reagent routinely produced maximal activation factors of 50-fold with 8-10-fold stimulation of maximum product activation levels. Activating power was totally dependent on the side chain length of the imidazolides. Short chain imidazolides either did not affect or produced some inhibition to the enzyme activity (Table 4). The explanation of this is that the short chain imidazolides might not be reactive with the activation site of the enzyme. The evidence for this is that, when the enzyme was treated first with acetyl imidazolidide and then with oleoyl imidazolidide, the oleoyl imidazolidide activated the enzyme by a factor of 21-fold (Fig 34). Furthermore, competitive experiments between lauroyl and oleoyl imidazolides showed that the oleoyl compound reacted more rapidly

with the enzyme in addition to producing higher activity levels (Fig 35). The relatively low activity produced by lauroyl imidazolide in comparison with the oleoyl residue seems to be due to the lower reactivity of the compound and not to lower effectiveness of the bound lauroyl residue. The rate of reactivity of the activator and hence its specificity increases as acyl side chain length increases. This pattern of activation is however in agreement with the activation produced by the free fatty acids.

Once more radiolabelling experiments using $[^{14}\text{C}]$ -labelled oleoyl imidazolide confirmed that the activator modifies the enzyme and not the substrate. Furthermore these results provided evidence that only one acyl group was added to each enzyme molecule to give full activation. There may be second highly reactive site for these reagents, or the additional residue may be added randomly to all susceptible groups on the protein. A low level of excess acylation has little inhibiting effect in this assay but may have more inhibiting effects in other more physiological assay systems.

Kinetic analysis showed that this activation is determined by an increase in the V_{max} with a small modification of K_m (Fig 39). Furthermore, enzyme activated by an optimum concentration of free oleic acid showed a similar kinetic behaviour with a V_{max} determined activation. These results combined with that acyl-group activation substitutes completely for fatty acid activation and also both acyl-group and free fatty acid activation affect the hydrolysis of similar types of substrates, suggest that acylating agents act by modifying the protein and also that fatty acids act by the same mechanism.

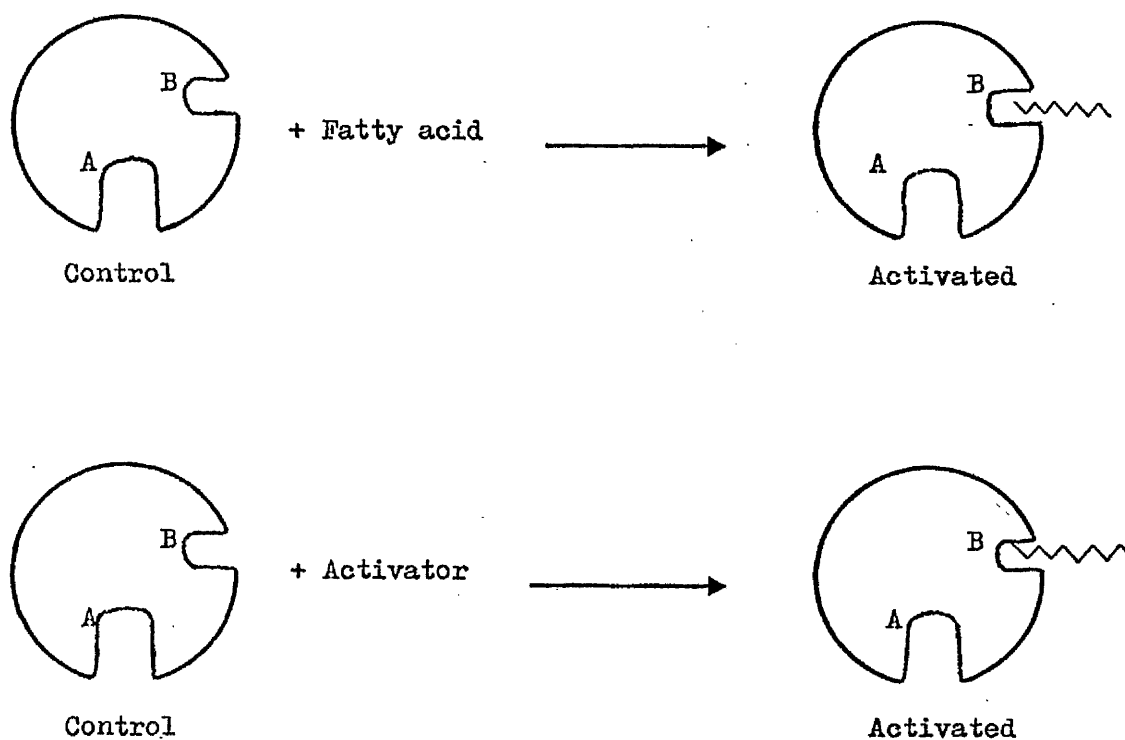


Fig (49) Model for the mechanism of PLA₂ activation by free acids and acylating agents.

A, represents the active site

B, represents the site where the activator binds.

A simple model can be proposed for this type of activation (Fig 49). The enzyme has two sites, the active site for the substrate and the activating site for the acyl residue. Free acids bind to the enzyme reversibly while acylating agents modify the protein in an irreversible way (Fig 49).

7.5 Possible Mechanisms of Activation

The possible explanations for the enzyme-mediated mechanism are as follows: a) the modified protein has increased its affinity for the substrate, b) the modified enzyme can penetrate more easily into the interface, c) the acyl group might cause allosteric modifications to the enzyme in such a way that the enzyme reacts more easily with the substrate, d) the acyl group might cause dimerization of the protein which increases its catalytic activity. The first explanation can be eliminated because increased affinity should change the value of the K_m parameter and this was not observed.

It is of interest to discuss at this point the effect of dimethyl maleic anhydride on the enzyme activity. This compound inactivates the non-activated and the covalently activated enzyme towards long chain substrates, but has no effect towards short chain substrates (Fig 26). Dimethyl maleic anhydride inactivates the non-activated enzyme by inhibiting the fast part of the hydrolysis curve of long chain substrates and gives a general inactivation to the covalently activated enzyme. A simple explanation for the DMMA action that one might suggest is that the compound simply blocks the

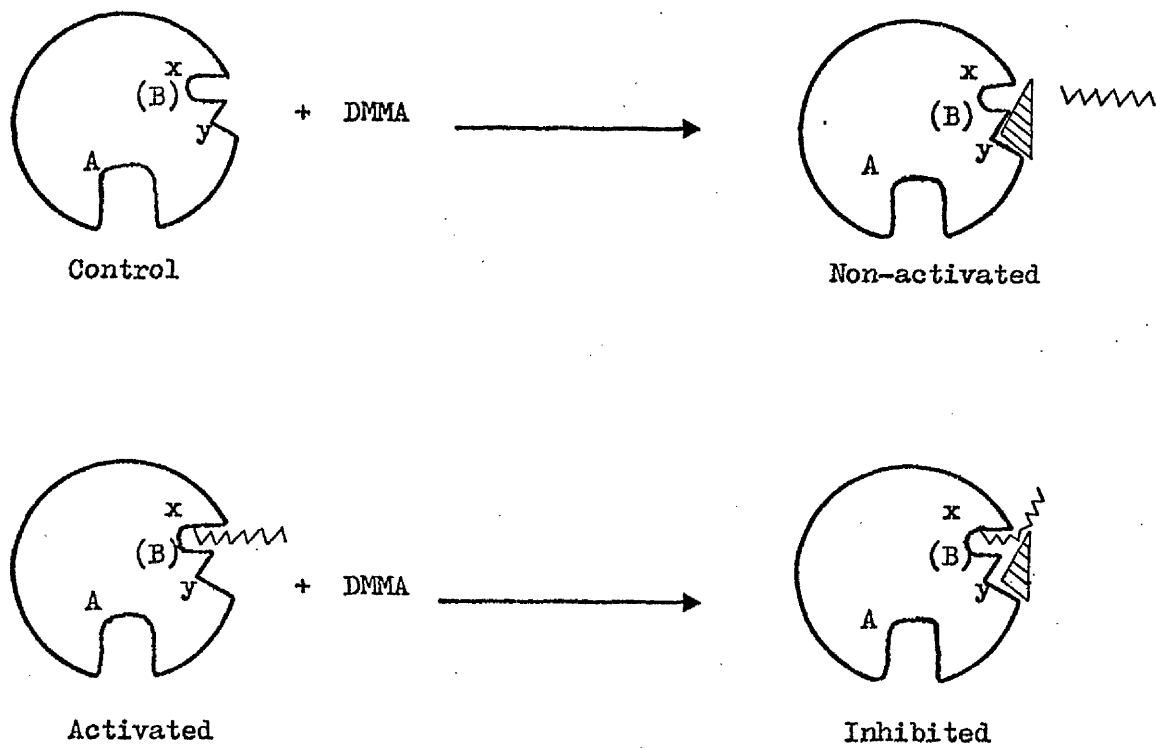


Fig (50) Model for the action of DMMA on the untreated and preactivated Bee venom PLA_2 .

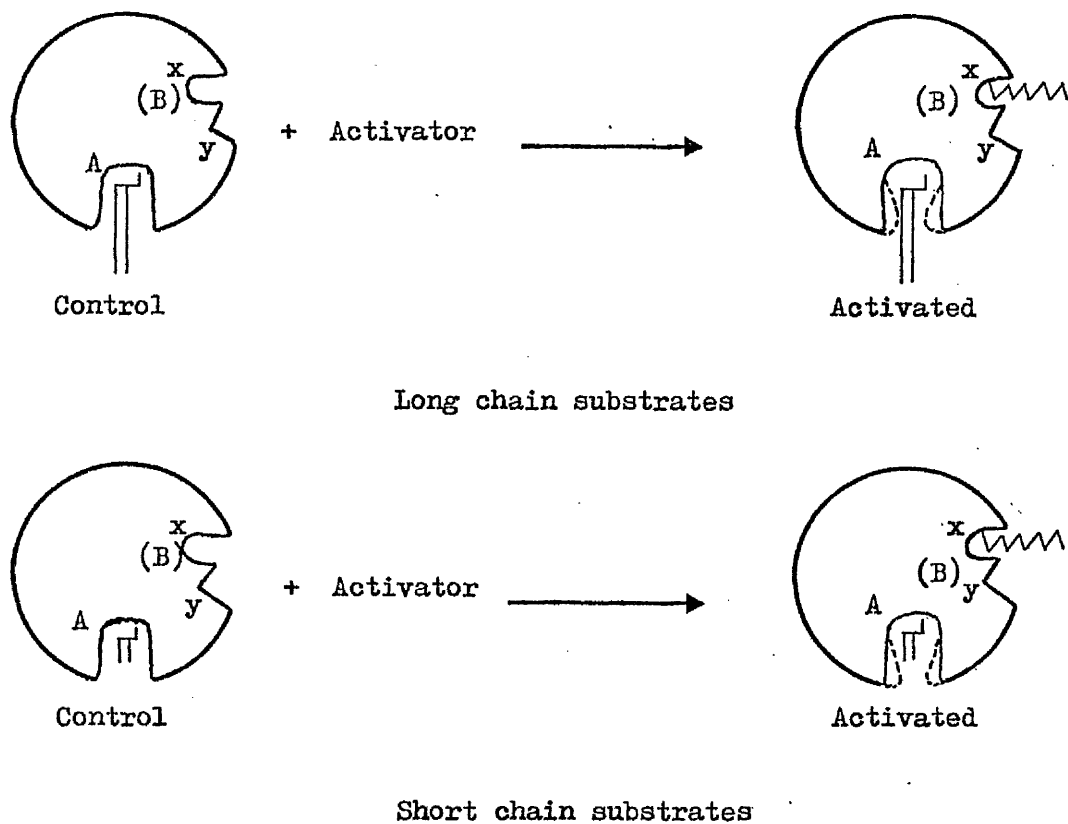


Fig (51) Model for the mechanism of action of preactivated
Bee venom PLA_2 on both long and short-chain substrates.

activating site of the enzyme, although this does not explain why DMMA inactivates the covalently activated enzyme and also why this inactivation does not affect the short chain substrates. One further possibility is that there are two nucleophilic groups at the activating centre and one of these (x) interacts with the activator, whilst the other (y) must remain free for the activating modification to be expressed. If DMMA binds to group (y) then interaction of the free or the covalently bound activator with group (x) might produce very little effect (Fig 50).

To explain why acylation or DMMA treatment of the enzyme does not affect its activity on short chain substrates, it is necessary to extend the previous model. A possible mechanism is that the acyl group might cause conformational changes in the enzyme, which make it more effective with the long-chain substrates but which do not work for short-chain compounds. The diagrams of the Fig 51 illustrate this possible mechanism. The conformational change caused by the acyl residue is therefore transmitted to the active site by means of the side chains of the substrate (Fig 51).

It was of theoretical interest here to speculate on the overall nature of the activation of the enzyme. On the one hand activation could be a positive effect whereby the catalytic efficiency of the enzyme is enhanced by the presence of the activator whilst on the other it might be that activation is the removal of a restraint present with long-chain substrates but absent with short-chain substrates. This latter system could have arisen as a self

protection mechanism so that membranes are not attacked unless the enzyme is specifically activated. The present results are not conclusive in distinguishing between these possibilities. Basically the enzyme attacks short-chain compounds more rapidly than long-chain compounds and activation simply raises the rate for the former group towards that seen for the latter group. This suggests that activation is the removal of a restraint. In contrast, the inhibition studies suggest that the unactivated state of the enzyme (for long-chain substrates) is equivalent to the normal state for short chain substrates. This suggests that activation is a positive effect. In order to resolve this problem further qualitative studies of the activity of activated and control enzyme on a variety of substrates will be required.

7.5.1 Enzyme Dimerisation

Another possible explanation is that the enzyme forms a dimer in order to hydrolyse long-chain substrates, while this is not necessary for its action on the more loosely packed short chain substrates. Therefore it is possible that activation might stabilise the dimeric form of the enzyme. DMMA could inhibit enzyme action by dissociating the dimers. This reagent attacks amino groups of the enzyme and could reverse the net positive charge causing dissociation into monomers, as found with the maleic anhydride (Sia and Horecker, 1968).

Further evidence to support this model might be obtained from the studies of PLA₂ activation in the aqueous assay systems.

Maximum activation should only be obtained when very low levels of activator were used. It is difficult to interpret the results adequately because of differences seen in the behaviour of the activated enzyme in the propanolic and in the aqueous assay systems. The enzyme may be activated in either medium and the time courses seem to be very similar. However the time course of response seems to differ in the different assay media. This tends to suggest that the requirements for activation are different in the different systems. Basically the results suggest that maximum activation in the propanolic medium corresponds to the addition of a single acyl residue to each protein molecule. If activation occurs more rapidly when the response is tested in the aqueous buffer, there are two possible explanations; either maximum activation corresponds to addition of one acyl residue to one monomer in a protein dimer, or the enzyme is far more sensitive to inhibition by non-specific acylation in the aqueous assay medium. The latter must be considered more probable because it is a simpler explanation. For example, if addition of a second residue is totally inhibiting in this system, activity would reach a maximum and then would decrease quite rapidly. However it is not unreasonable to propose that the enzyme might dimerise in an aqueous medium, but exist as monomers in a medium which weakens hydrophobic interaction. However, if dimer formation is the basis of activation in the aqueous medium and entirely different basis for activation would be required for the propanolic system, therefore it is reasonable to dismiss this mechanism unless further evidence is obtained in its support.

This possibility that Bee venom PLA₂ acts as a dimer must be considered because of compelling evidence that is true for other similar enzymes. Roberts et al (1977) proposed that the cobra venom Naja-Naja-Naja PLA₂ acts as a dimer because it could be inhibited by addition of one molecule of p-bromophenacyl bromide to two molecules of protein. Wells (1971) provided evidence that the Crotalus adamanteus PLA₂ acts as a dimer and also Shipolini et al (1971) reported that the Bee venom PLA₂ exists in concentrated solution at high pH as a dimer.

7.5.2 The Interfacial Penetration Theory

Another attractive mechanism is that the activator facilitates functional penetration of the lipid interface by the enzyme. Such a rate limiting process has been determined for many phospholipase A enzymes by a monolayer assay (Verger et al, 1973). Verger et al (1973) in order to describe the kinetics of the action of soluble enzymes at interfaces, proposed a simple model which consists of two successive equilibria. Firstly the slow reversible penetration of the enzyme (E) into an interface takes place. When the enzyme has penetrated, it is in a more favourable energetical state (E'). After this first step of penetration the enzyme reacts according to the classical theory (Verger et al, 1973). Also Verger et al (1973) demonstrated induction times for bee venom PLA₂ towards monolayers of dinonanoyl lecithin but only at high film pressures. In contrast the pancreatic enzyme only acts at much lower surface pressures. They suggested that the more efficient penetration site of the bee venom PLA₂ might have arisen because it normally has to penetrate the condensed phospholipid architecture of biological

membranes; in contrast the pancreatic enzyme does not require such an efficient penetration site, because this enzyme normally acts under conditions where natural detergents like bile salts are present. The evidence that led Verger et al (1973) to propose the penetration mechanism (obtained with the pancreatic enzyme) is: the induction time was independent of enzyme concentration; the velocity was proportional to enzyme concentration; the lag period was not due to a slow homogenization of the injected enzyme below the surface film because complete mixing was attained in less than 30 sec, and finally the possibility that the acceleration of the enzymatic velocity was due to the presence of increasing amounts of reaction products at the interface was eliminated because for the short chain substrates there is a rapid desorption of both lysophospholipids and fatty acids from the surface film into the aqueous subphase. In contrast with this evidence the present results suggest that the penetration step cannot be rate limiting, as the hydrolysis curve for non-activated enzyme shows significant activity but is convincingly linear for a long time period (Fig 47), also the lag period is dependent on the enzyme concentration, and finally there is product activation for the bee venom enzyme. These results suggest that it is more likely that the Bee venom PLA₂ does not follow a penetration mechanism to attack interfaces.

7.5.3 Summary

The model of allosteric activation of a protein monomer appears to be the simplest and most satisfactory of all those discussed

above and is the only one which can account for all the experimental results without complicated additional hypotheses.

7.6 Relationship of Calcium Activation and Fatty Acid Activation

Another interesting characteristic of the covalently activated enzyme lies in the requirement of calcium. The results confirmed that the enzyme has two sites for calcium, one with high affinity which is normally saturated, and one with low affinity which is responsible for ca 8-fold increase of activity of the normal enzyme. The activated enzyme is much less sensitive to the second calcium ion than the normal enzyme and, indeed, fully activated may have no requirement for ion binding at the second site. This is supported by the fact that the activated enzyme is less sensitive to strontium inhibition and it might be probable that the completely activated enzyme is not in any way sensitive to strontium inhibition. It would be of interest to design calcium binding experiments to find if the low affinity site is related with the fatty acid site because the results suggest that the fatty acid might substitute the second weakly reactive calcium. There is difficulty with the simple interpretation which is apparent when the substrate structure is considered. The non-activated enzyme is sensitive to calcium with all types of substrates, therefore the calcium binding site must be more closely related to the active site than is the fatty acid binding site.

7.7 Specification of Activation

Interest in the chemistry of the activation lies in the factors which determine specificity for the activation site. There may be a small degree of chemical specificity, for example, imidazolides react more readily with tyrosyl than with lysyl residues (Riordan et al, 1965), but after treatment of the activated enzyme with 1 M hydroxylamine to reverse the activation (Riordan et al, 1965) the enzyme was still activated. The only way to find out which amino acid is responsible for the activation is peptide mapping analysis of the covalently activated enzyme.

7.8 Other Implications

Preliminary tests show that the phospholipases A₂ from Vipera ammodytes venom and from Naja-Naja venom are indeed activated by oleoyl imidazolidide although the effects are not as large as those seen with the Bee venom enzyme. These results suggest that the mechanism of bee venom PLA₂ activation with acyl-imidazolides might be a general mechanism and the agents most active in the present system could also be irreversible activators for many other fatty acid activated enzymes.

The chemical properties of acyl imidazolides make them ideal for use as phospholipase activators in cells or tissues. They have low general reactivity and are sufficiently hydrophilic to activate the enzyme in a purely aqueous incubation system and should therefore

reach enzymes at any site within the cells.

Finally, fatty acids are membrane-active and also interact with calcium, the dominant activator in the system. Therefore the use of irreversible activators which are not charged or membrane active, provides the first opportunity for distinguishing the different effects of fatty acids in cell membranes.

REFERENCES

- AARSMAN, A.J., VAN DEENEN, L.L.M. AND VAN DEN BOSCH, H. (1976)
Studies on Lysophospholipases. VII. Synthesis of Acylthioester
Analogues of Lysolecithin and their Use in a continuous Spectro-
photometric Assay for Lysophospholipases, a Method with Potential
Applicability to Other Lipolytic Enzymes.
Bioorganic Chemistry 5: 241-253.
- ALBERTSON, N.F. (1962)
Synthesis of Peptides with Mixed Anhydrides.
Organic Reactions vol 12: 157-355.
- ANSELL, G.B. AND HAWTHORNE, J.N. (1964)
in "Phospholipids".
Elsevier, Amsterdam.
- BANGHAM, A.D. AND DAWSON, R.M.C. (1962)
Electrokinetic Requirements for the Reaction between Cl. perfringens
 α -Toxin (Phospholipase C) and Phospholipid Substrates.
Biochimica et Biophysica Acta 59: 103-115.
- BANGHAM, A.D. AND DAWSON, R.M.C. (1960)
The Physicochemical Requirements for the Action of Penicillium
notatum Phospholipase B on Unimolecular Films of Lecithin.
Biochemical Journal 75: 133-138.
- BIRD, PH.R., DE HAAS, G.H., HEEMSKERK, T.H.TH. AND VAN DEENEN, L.L.M.
(1965)
Synthetic Lecithins containing one short-chain Fatty Acid and
their Breakdown by Phospholipase A.
Biochimica et Biophysica Acta 98: 566-573.

- BLASCHKO, H., SMITH, A.D., WINKLER, H., VAN DEN BOSCH, H. AND VAN DEENEN, L.L.M. (1967)
 Acid Phospholipase A in Lysosomes of the Bovine Adrenal Medulla.
 Biochemical Journal 103: 30c.
- BONSEN, P.P.M., PIETERSON, W.A., VOLWERK, J.J. AND DE HAAS, G.H. (1971)
 Phospholipase A and its Zymogen from Porcine pancreas.
 Biochemical Society Symposium 35: 189-200.
- BONSEN, P.P.M., DE HAAS, G.H., PIETERSON, W.A. AND VAN DEENEN, L.L.M. (1972)
 Studies on Phospholipase A and its Zymogen from Porcine pancreas.
 IV. The Influence of Chemical Modification of the Lecithin structure on substrate properties.
 Biochimica et Biophysica Acta 270: 364-382.
- VAN DEN BOSCH, H. (1974)
 Phosphoglyceride Metabolism.
 Annual Review of Biochemistry 43: 243-277.
- BROKERHOF, H. AND YURKOWSKI, M. (1965)
 Simplified Preparation of L- α -Glyceryl Phosphoryl Choline.
 Canadian Journal of Biochemistry 43: 1777.
- BUTLER, P.J.G., HARRIS, J.I., HARTLEY, B.S. AND LEBERMAN, R. (1967)
 Reversible Blocking of peptide Amino Groups by Maleic Anhydride.
 Biochemical Journal 103: 78p.
- CURTIS, A.S.G., CAMPBELL, J. AND SHAW, F.M. (1975)
 Cell Surface Lipids and Adhesion. I. The effects of lyso-phosphatidyl Compounds, Phospholipase A₂ and Aggregation-Inhibiting Protein.
 Journal of Cell Science 18: 347-356.

CURTIS, A.S.G., SHAW, F.M. AND SPIRES, W.M.C. (1975)

Cell Surface Lipids and Adhesion. II. The Turnover of Lipid
Components of the Plasmalemma in Relation to Cell Adhesion.
Journal of Cell Science 18: 357-373.

DAWSON, R.M.C. (1963)

On the Mechanism of Action of Phospholipase A.
Biochemical Journal 88: 414-423.

DAWSON, R.M.C. (1958)

Studies on the Hydrolysis of Lecithin by a *Penicillium notatum*
Phospholipase B Preparation.
Biochemistry 70: 559-570.

DAWSON, R.M.C. AND BANGHAM, A.D. (1959)

The Activation of Surface Films of Lecithin by Amphipathic
Molecules.
Biochemical Journal 72: 493-496.

DEEMS, R.A. AND DENNIS, E.A. (1975)

Characterization and Physical Properties of the Major Form of
Phospholipase A₂ from cobra venom (*Naja naja naja*) That has a
Molecular Weight of 11,000.
Journal of Biological Chemistry 250: 9008-9012.

VAN DEENEN, L.L.M. AND DE HAAS, G.H. (1963)

The Substrate Specificity of Phospholipase A.
Biochimica et Biophysica Acta 70: 538-553.

VAN DEENEN, L.L.M., DE HAAS, G.H. AND HEEMSKERK, C.H.TH. (1963)

Hydrolysis of Synthetic Mixed Acid Phosphatides by Phospholipase A
from Human Pancreas.
Biochimica et Biophysica Acta 67: 295-304.

EPSTEIN, B. AND SHAPIRO, B. (1959)

Lecithinase and Lysolecithinase of Intestinal Mucosa.

Biochemical Journal 71: 615-619.

FIESER AND FIESER (1967)

Reagents for Organic Synthesis vol. 1: 1041-1042.

FISCHER, H., FERBER, E., HAUPT, I., KOHLSCHÜTTER, A., MODOLELL, M.,

MUNDER, P.G. AND SONAK, R. (1967)

Lysophosphatides and Cell Membranes.

Protides of the Biological Fluids 15: 175-184.

FRANSON, R., WAITE, M. AND LA VIA, M. (1971)

Identification of Phospholipase A₁ and A₂ in the Soluble Fraction
of Rat liver Lysosomes.

Biochemistry 10: 1942-1946.

GATT, S. AND BARENHOLZ, Y. (1973)

Enzymes of Complex Lipid Metabolism.

Annual Review of Biochemistry 42: 61-90.

DE HAAS, G.H., POSTEMA, N.M., NIEUWENHUIZEN, W. AND VAN DEENEN, L.L.M.

(1968)

Purification and Properties of Phospholipase A from Porcine Pancreas.

Biochimica et Biophysica Acta 159: 103-117.

DE HAAS, G.H., BONSEN, P.P.M., PIETPERSON, W.A. AND VAN DEENEN, L.L.M.

(1971)

Studies on Phospholipase A and its Zymogen from Porcine pancreas.

III. Action of the Enzyme on Short-Chain Lecithins.

Biochimica et Biophysica Acta 239: 252-266.

HABERMANN, E. (1972)

Bee and Wasp Venoms.

Science 177: 314-322.

HANSS, M. AND REY, A. (1971)

Application de la Conductimétrie a l'étude des Réactions
enzymatiques. Butyrylcholinestérase.

Biochimica et Biophysica Acta 227: 618-629.

HENRI, V. AND DES BANCELS, L. (1903)

Étude de la digestion de la caséine par la méthode de
conductibilité électrique.

C.R. Séances Soc. Biol. 4: 563.

OP DEN KAMP, J.A.F., DE GIER, J. AND VAN DEENEN, L.L.M. (1974)

Hydrolysis of Phosphatidylcholine liposomes by Pancreatic
phospholipase A₂ At the transition Temperature.

Biochimica et Biophysica Acta 345: 253-256.

LAWRENCE, A.J. (1971)

Conductimetric Enzyme Assays.

European Journal of Biochemistry 18: 221-225.

LAWRENCE, A.J. AND MOORES, G.R. (1972)

Conductimetry in Enzyme Studies.

European Journal of Biochemistry 24: 538-546.

LAWRENCE, A.J., MOORES, G.R. AND STEELE, J. (1974)

A Conductimetric Study of Erythrocyte Lysis by Lysolecithin and
Linoleic Acid.

European Journal of Biochemistry 48: 277-286.

LAWRENCE, A.J. (1975)

Lysolecithin inhibits an action of Bee venom Phospholipase A₂
in Erythrocyte Membrane.

Febs Letters 58: 186-189.

LAWRENCE, A.J. AND MOORES, G.R. (1975)

Activation of Bee venom Phospholipase A₂ By Fatty acids

Aliphatic Anhydrides and Glutaraldehyde.

Febs Letters 49: 287-291.

LONG, C. AND PENNY, I.F. (1957).

The structure of the Naturally occurring Phosphoglycerides.

Biochemical Journal 65: 382-389.

LUCY, J.A. (1970)

The Fusion of Biological Membranes.

Nature 227: 814-817.

MENZEL, D.B. AND OLCOTT, H.S. (1964)

Positional Distribution of Fatty Acids in Fish and other Animal
Lecithins.

Biochimica et Biophysica Acta 84: 133-139.

NAIR, C., BERMANS, J., MUNJAL, D. AND ELLIOTT, W.B. (1976)

Temperature Stability of Phospholipase A Activity - I, Bee

(Apis Mellifera) venom Phospholipase A₂.

Toxicon 14: 35-42.

NAIR, B.C., NAIR, C. AND ELLIOTT, W.B. (1976)

Temperature Stability of Phospholipase A Activity - II, Variations
in Optimum Temperature of Phospholipases A₂ from Various Snake
Venoms.

Toxicon 14: 43-47.

NEUMANN, W. AND HABERMANN, E. (1954)

"
Beitrage zur Charakterisierung der Wirkstoffe des Bienengiftes.

Arch. exper. Path. Pharmacol. 222: 367-387.

NEWKIRK, J.D. AND WAITE, M. (1971)

Identification of a Phospholipase A_1 in plasma Membranes.

Biochimica et Biophysica Acta 225: 224-233.

OWEN, M.D. (1971)

Insect Venoms: Identification of Dopamine and Noradronaline
in Wasp and Bee Stings.

Experientia 27: 544-545.

PIETERSON, W.A., VIDAL, J.C., VOLWERK, J.J. AND DE HAAS, G.H. (1974) x

Zymogen-Catalyzed Hydrolysis of Monomeric substrates and the
Presence of a Recognition site for Lipid-Water Interfaces in
phospholipase A_2 .

Biochemistry 13: 1455-1460.

RIGGS, M.G., WHITTAKER, R.G., NEUMANN, J.R. AND INGRAM, V.M. (1977)

n-Butyrate causes histone modification in HeLa and Friend
erythroleukaemia cells.

Nature 268: 462-464.

RIORDAN, J.F., WALKER, W.E.C. AND VALLEE, B.L. (1965)

N-Acetylimidazole: A Reagent for Determination of "Free"
Tyrosyl Residues of Proteins.

Biochemistry 4: 1758-1765.

ROBERTS, M.F., DEEMS, R.A. AND DENNIS, E.A. (1977)

Dual role of interfacial Phospholipid in Phospholipase A_2 Catalysis.

Proc. Natl. Acad. Sci. USA, 74: 1950-1954.

ROBERTS, M.F., DEEMS, R.A., MINCEY, T.C. AND DENNIS, E.A. (1977)

Chemical modification of the histidine residue in phospholipase A_2

(Naja-Naja-Naja): A case of half-site reactivity.

Journal of Biological Chemistry 252: 2405-2411.

ROHOLT, O.A. AND SCHLAMOVITZ, M. (1961)

Studies of the Use of Dihexanoyllecithin and other Lecithins as Substrates for Phospholipase A with Addendum on Aspects of Micelle properties of Dihexanoyllecithin.

Archives in Biochemistry and Biophysics 94: 364-379.

SCHACHTER, M. AND THAIN, E.M. (1954)

Chemical and Pharmacological properties of the Potent, Slow Contracting Substance (KININ) in Wasp Venom.

British Journal of Pharmacology 9: 352-359.

SELINGER, Z. AND LAPIDOT, Y. (1966)

Synthesis of Fatty acid Anhydrides by reaction with dicyclohexylcarbodiimide.

Journal of Lipid Research 7: 174-175.

SHIPOLINI, R.A., CALLEWAERT, G.L., COTTRELL, R.C., DOONAN, S.,

VERNON, C.A. AND BANKS, B.E.C. (1971)

Phospholipase A from Bee Venom.

European Journal of Biochemistry 20: 459-468.

SHIPOLINI, R.A., CALLEWAERT, G.L., COTTRELL, R.C. AND VERNON, C.A.

(1971)

The primary sequence of phospholipase-A from Bee Venom.

Febs letters 17: 39-40.

SHIPOLINI, R.A., CALLEWAERT, G.L., COTTRELL, R.C. AND VERNON, C.A.

(1974)

The Amino-Acid Sequence and Carbohydrate Content of Phospholipase A₂ from Bee Venom.

European Journal of Biochemistry 48: 465-476.

SHIPOLINI, R.A., DOONAN, S. AND VERNON, C.A. (1974)

The Disulphide Bridges of Phospholipase A₂ from Bee Venom.

European Journal of Biochemistry 48: 477-483.

SIA, C.L. AND HORECKER, B.L. (1968)

Dissociation of Protein Subunits by Maleylation.

Biochemical and Biophysical Research Communications 31: 731-737.

SIMPSON, R.T., RIORDAN, J.F. AND VALLEE, B.L. (1963)

Functional Tyrosyl Residues in the Active Center of Bovine
Pancreatic Carboxypeptidase A.

Biochemistry 2: 616-621.

STERN, I. AND SHAPIRO, B. (1953)

A Rapid and Simple Method for the Determination of Esterified
Fatty Acids and for Total Fatty Acids in Blood.

Journal of Clinical Pathology 6: 158-160.

STODDART, R.W. AND PRICE, M.R. (1976)

The Comparison of Membrane Proteins of Normal and Neoplastic
Cells by Isoelectric Focusing.

Biochemical Society Transactions 4: 675-677.

SU-CHEN, L.H. AND LEVINE, L. (1976)

Inhibition of Arachidonic acid release from cells as the
biochemical action of anti-inflammatory corticosteroids.

Proc. Natl. Acad. Sci. USA. 73: 1730-1734.

VERGER, R., MIERAS, M.C.E. AND DE HAAS, G.H. (1973)

Action of Phospholipase A at Interfaces.

The Journal of Biological Chemistry 248: 4023-4034.

VOLWERK, J.J., PIETERSON, W.A. AND DE HAAS, G.H. (1974)

Histidine at the Active Site of Phospholipase A₂.

Biochemistry 13: 1446-1454.

WAITE, M. AND VAN DEENEN, L.L.M. (1967)

Hydrolysis of phospholipids and Glycerides by Rat-Liver preparations.

Biochimica et Biophysica Acta 137: 498-517.

WAITE, M., VAN DEENEN, L.L.M., RUIGROK, T.J.C. AND ELBERS, P.F.

(1969)

Relation of Mitochondrial Phospholipase A activity to Mitochondrial Swelling.

Journal of Lipid Research 10: 599-608.

WAITE, M., SCHERPHOF, G.L., BOSHOEWERS, F.M.G. AND VAN DEENEN, L.L.M.

(1969)

Differentiation of Phospholipases A in mitochondria and lysosomes of rat liver.

Journal of Lipid Research 10: 411-420.

WELLS, M.A. (1971)

Evidence that the Phospholipase A₂ off Crotalus adamanteus

Venom are Dimers.

Biochemistry 10: 4074-4078.

WELLS, M.A. (1972)

A Kinetic Study of the Phospholipase A₂ (Crotalus adamanteus)

Catalyzed Hydrolysis of 1,2-Dibutyryl-sn-glycero-3-phosphorylcholine.

Biochemistry 11: 1030-1041.

WELLS, M.A. (1973)

Effects of chemical Modification on the Activity of Crotalus
adamanteus Phospholipase A₂. Evidence for an Essential Amino
Group.

Biochemistry 12: 1086-1093.

WELLS, M.A. (1973)

Spectral Perturbations of Crotalus adamanteus Phospholipase A₂
Induced by Divalent Cation Binding .

Biochemistry 12: 1080-1085.

WELLS, M.A. (1974)

The Mechanism of Interfacial Activation of Phospholipase A₂.

Biochemistry 13: 2248-2257.

WELLS, M.A. AND HANAHAN, D.J. (1969)

Studies on phospholipase A. Isolation and Characterization of
two Enzymes from Crotalus adamanteus Venom.

Biochemistry 8: 414-424.